Studies on *Brucella ovis* infection in deer

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Abstract

Brucella ovis was first identified in the New Zealand farmed deer population in 1996 but little was known about the disease in deer. These experiments were undertaken to investigate the epidemiology, pathophysiology and diagnosis of *B. ovis* infection in deer. In addition, *B. ovis* isolates from commercial rams and stags were strain typed by pulsed-field gel electrophoresis.

Transmission of infection was demonstrated from infected rams to stags grazing in the same paddock, suggesting that the initial source of infection for deer in New Zealand was likely to have been from rams. Transmission between stags did not occur after shifting non-infected stags into paddocks immediately vacated by infected stags, or after grazing non-infected stags in a paddock adjacent to infected stags over a five and a half month period. This suggests that the risk of transmission of *B. ovis* by the environment or indirect deer to deer contact is low. Stags became infected with *B. ovis* after experimental inoculation of the conjunctival, nasal and rectal mucous membranes. Behavioural observations identified that stags in all-male groups interact by mounting, sniffing the prepuce and perineum and spraying fluid from an extruded penis, which are considered high risk for the transmission of *B. ovis*.

It was established that while stags are initially as susceptible to *B. ovis* infection as rams the majority of stags stop shedding *B. ovis* in semen within 11 months of infection, suggesting resolution of infection. In contrast, all rams remained infected with *B. ovis* and shed the organism in semen for at least 21 months.

During the *B. ovis* shedding phase of infection, the majority of stags produced semen that had poor sperm motility and morphology and contained large numbers of leukocytes and cellular debris. However, following cessation of shedding stags produced semen that had good sperm motility and morphology, although leukocytes were still present.

The sensitivity of the commercially available serological tests at detecting infection in deer was 100% during the early stages of the disease but after 60 to 100 days of infection, their sensitivity decreased to 30 to 70%. In contrast, the sensitivity in rams

over a 630-day period was 100%. Detection of lesions of epididymitis by scrotal palpation of stags was an insensitive method of diagnosing infection.

Stags infected with *B. ovis* developed lesions in the epididymes, seminal vesicles and ampullae similar to those reported in rams. In the early stages of the infection, lesions in stags were severe but in more chronic infections the lesions were mild.

Vaginal inoculation of hinds immediately prior to mating resulted in no measurable adverse effects on reproduction, suggesting the disease is of little significance in hinds. Stags that mated vaginally-infected hinds became infected, demonstrating venereal transmission of the organism.

Pulsed-field gel electrophoresis of *B. ovis* isolates revealed the presence of two strain types of *B. ovis* in the New Zealand farmed sheep and deer populations. Cervine isolates from two naturally-occurring cases of *B. ovis* in stags were different strain types. This confirmed that the two cases were unrelated, again highlighting the importance of rams in the epidemiology of this disease in deer.

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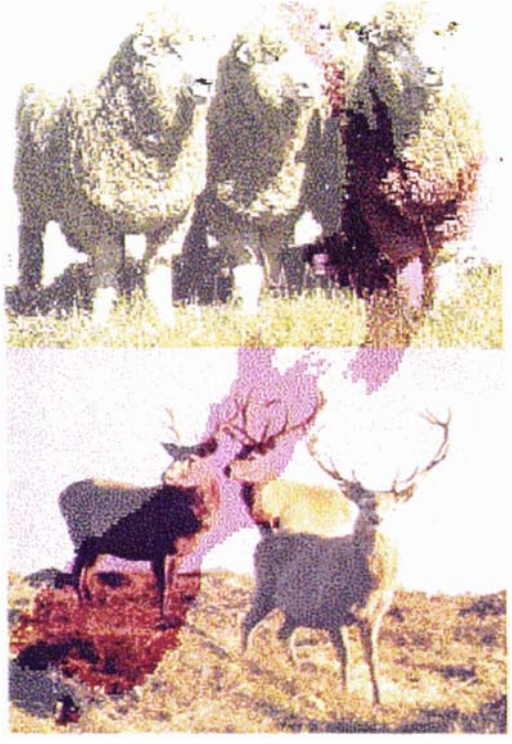
An overview of *Brucella ovis* infection in sheep and deer with particular reference to New Zealand



A summarised version of aspects of this literature review, entitled "An overview of *Brucella ovis* in New Zealand" by AL Ridler, has been accepted by the New Zealand Veterinary Journal and is in press.

1.1 Introduction

A report from the 1950 lambing season in the Gisborne region of New Zealand documented a Gram negative cocco-bacillus, described as a "Rickettsia-like organism" causing third-trimester abortion in over 50 of a flock of 1100 ewes (McFarlane et al., 1952). An addendum to this paper reported that a similar organism had also been isolated from rams with epididymitis (McFarlane et al., 1952). The organism was thought to resemble the agent causing enzootic abortion in the United Kingdom reported on by Stamp et al. (1950) as it was acid fast when stained with the modified Ziehl-Neelson stain. However, after personal communication with Stamp the authors concluded that this was a different organism. In 1953 the same organism that was identified in Gisborne was identified causing epididymitis in rams in New Zealand and Australia (Buddle and Boyes, 1953; Simmons and Hall, 1953). The organism was subsequently characterised as being of the *Brucella* species and named *Brucella ovis* (Buddle, 1956). The disease it causes in sheep is commonly referred to as ovine brucellosis.

Brucella ovis is a Gram negative cocco-bacillus which, in contrast to other *Brucellae*, is not zoonotic. The organism predominantly resides in the genital tract of infected males. Strain typing using Restriction Endonuclease techniques have demonstrated only one detectable strain-type (Bailey and West, 1987a; O'Hara et al., 1985).

1.2 Epidemiology of infection in sheep

1.2.1 Host range

Brucella ovis is a disease primarily of sheep and has been reported in sheep flocks from most parts of the world with the exception of Great Britain (Lawrence, 1961). There is a single report of suspected natural infection in wild deer in the United States of America (Barron, 1984) and in 1996 infection was reported in a stag in New Zealand (O'Neil, 1996). There are no reports of natural infection occurring in any other species.

Experimentally, efforts have been made to infect a variety of different species with *B. ovis*. Buddle and Boyes (1953) inoculated two five-month-pregnant cows by intravenous injection of *B. ovis* organisms. One cow was slaughtered one week prior to calving and the other was allowed to calve normally. There was no evidence of infection in the foetal membranes or the calves, but *B. ovis* was isolated from the milk of the cow that was not slaughtered.

Burgess et al. (1985) inoculated male goats with infected semen by either the intratesticular, intrapreputial or intranasal routes, with two goats inoculated by each route. All six goats developed serological titres. Up to the time of slaughter on day 98, *B. ovis* was cultured from the semen from both goats inoculated by the intratesticular route and from one inoculated by the intranasal route. It was reported that the infected goats were shedding the organism in semen in very low numbers by the end of the 98-day period but this finding was not quantified.

Artificial infection of guinea pigs, rabbits, mice, rats, hamsters and gerbils by either the intratesticular, intraperitoneal or intravenous routes have been undertaken (Buddle and Boyes, 1953; Cuba-Caparo and Myers, 1973). All species had serological titres in both a *B. ovis* complement fixation test and an agar-gel test at variable times after inoculation and at slaughter all had *B. ovis* isolated from some or all of the liver, lung, spleen and testicle. Gerbils developed pathological lesions in the epididymes that closely resembled lesions seen in infected rams (Cuba-Caparo and Myers, 1973).

1.2.2 Transmission

Transmission of *B. ovis* can occur between rams in direct contact with one another (Buddle, 1955) but the precise mechanism of transmission has not been defined. The discovery of faeces in the prepuce of rams led to the theory that a common route of transmission of disease between rams is by homosexual activity including rectal copulation (Jebson et al., 1954; Hartley et al., 1955; Snowden, 1958). However, the evidence for this is circumstantial and it has not been demonstrated experimentally. Ejaculation onto the perineal region and subsequent sniffing or licking of the infected

semen by another ram has also been suggested as an important route of transmission (Keogh et al., 1958).

Experimentally, rams have been artificially infected with *B. ovis* by inoculation of either infected semen or suspended *B. ovis* cultures onto the conjunctival, nasal, oral, preputial/penile and rectal mucus membranes, or by injection of organisms intravenously or directly into the testes. A summary of these experiments is detailed in Table 1.2.1. Rams have been experimentally infected by shearing with hand-pieces artificially infected with *B. ovis* (Clapp, 1962).

Venereal transmission from ram to ram by ewes acting as a mechanical vector has been demonstrated (Hartley et al., 1955; Watts, 1955; Snowden, 1958). In these reports, rams became infected by mating ewes immediately after they had been mated by an infected ram. Van Rensburg et al. (1958) allowed a ewe to be mated by an infected ram, and at the next oestrus period took vaginal mucous from the ewe and applied it to the glans penis of a non-infected ram. This ram became infected, suggesting that rams may become infected by mating ewes that were mated by infected rams in the previous oestrus period. Buddle (1955) and Hartley et al. (1955) both mated rams to ewes that had *B. ovis*-induced placentitis in the previous lambing season. In one of these experiments four rams were mated to 27 ewes. Two of the four rams developed serological evidence of infection, but *B. ovis* was not isolated from semen or reproductive organs of any (Buddle, 1955). In the other experiment two rams were mated to nine ewes with neither ram becoming infected with *B. ovis* (Hartley et al., 1955).

Several experiments have been conducted to establish whether rams and ewes can become infected by grazing pastures recently stocked by infected animals. Keogh et al. (1958) alternated two groups of rams between two paddocks each week for a 12-month period. One group contained 15 infected and 19 non-infected rams and the second group contained 20 non-infected rams. Two rams in the first group, which had direct contact with infected rams, became infected with *B. ovis* but none of the rams in the second group became infected. Hartley et al. (1955) used 10 rams to graze lambing paddocks which had recently been vacated by artificially infected ewes. The ewes

lambing in these paddocks had infected placentae and were reported to have viable *B. ovis* organisms in vaginal discharges for at least 10 days after parturition, which could have led to contamination of the pasture. None of the 10 rams developed epididymitis or had *B. ovis* cultured from semen. Buddle (1955) grazed 10 ewes on pastures that had recently been stocked by 64 artificially infected ewes with gross lesions of *B. ovis*-induced placentitis at parturition. None of the 10 ewes showed clinical evidence of infection at lambing.

There are no published reports of transmission between infected and non-infected rams when grazing in adjacent paddocks. This evidence would suggest that transmission of *B. ovis* requires direct contact between animals.

Table 1.2.1 Experimental infection of rams with *B. ovis* by a variety of routes using either infected semen or a suspended culture of *B. ovis*, and success of infection measured by development of a serological titre, bacteriological culture of semen or reproductive organs, and development of lesions of epididymitis. n/r denotes not reported.

Route of inoculation	Type of inoculum	Dose (colony forming units)	Number of animals exposed	Number (and percentage) developing a serological titre	Number (and percentage) culture positive	Number (and percentage) developing epididymitis	Reference
Conjunctival	Culture	$1x10^9$	30[1]	n/r	25 (83)	n/r	Biberstein et al., 1964
	Culture	$3.5x10^8$	41	41 (100)	3 (7)	n/r	Brown et al., 1973
	Culture	$3.5x10^{10}$	7	7 (100)	5 (71)	n/r	Paolicchi et al., 2000
Nasal	Semen	n/r	15	11 (73)	3 (20)	n/r	Burgess and Norris, 1982
	Semen	$1x10^8$	9	9 (100)	3 (33)	3 (33)	Plant et al., 1986
Oral	Culture	n/r	2	n/r	0	1 (50)	Simmons and Hall, 1953
Prepuce/ penis	Culture	$1x10^8$	10	n/r	7 (70)	n/r	Buddle and Boyes, 1953
	Culture	$1x10^8$	10	n/r	7 (70)	8 (80)	Jebson et al., 1954
	Semen	n/r	25	17 (68)	6 (24)	7 (28)	Laws et al., 1972
	Culture	$1.5x10^{10}$	10	10 (100)	10 (100)	n/r	Webb et al., 1980
	Semen	$1x10^8$	9	9 (100)	1 (11)	1 (11)	Plant et al., 1986
	Culture	$1x10^8$	5	5 (100)	1 (20)	1 (20)	Plant et al., 1986
	Culture	$3.5x10^{10}$	7	7 (100)	4 (57)	n/r	Paolicchi et al., 2000
Rectal	Semen	$1x10^8$	9	6 (67%)	1 (11)	1(11)	Plant et al., 1986
Testicular	Culture	$1x10^8$	10	n/r	9 (90)	n/r	Buddle and Boyes, 1953
	Culture	n/r	14	14 (100)	11 (79)	n/r	Myers and Siniuk, 1970
Intravenous	Culture	$1x10^8$	20	n/r	18 (90)	n/r	Buddle and Boyes, 1953
	Culture	$1x10^9$	20	n/r	19 (95)	20 (100)	Jebson et al., 1954
	Culture	$1x10^7$	10	8 (80)	8 (80)	n/r	Ris, 1964
	Culture	$2.5x10^9$	11	11 (100)	5 (45)	5 (45)	Claxton, 1968
	Culture	$1.3x10^8$	5	4 (80)	4 (80)	4 (80)	Bailey, 1986

[1] In this trial, 48 rams were inoculated and then killed in pairs every twp to 14 days. Only rams examined at or after day 45, which was the first time *B. ovis* was isolated from the semen of rams, are included in this analysis

1.2.3 Environmental viability

The ability of *B. ovis* to survive in the environment, outside a host animal, has not been reported. Buddle (1957a) during a question and answer session at a farmer's meeting, stated that "under the most favourable conditions, the organisms can survive in infected material, such as foetal membranes, on pasture for months...". There does not appear to be any published evidence to substantiate this claim and it is possible that these environmental viability figures may have been extrapolated from *Brucella abortus* which has been reported to survive for at least two months in soil (Cameron, 1932). *Brucella ovis* has been demonstrated to survive in semen for at least three days at room temperature (Southern, 1983).

1.3 Pathophysiology of *B. ovis* infection in rams

1.3.1 Pathogenesis

Most of the work investigating the pathogenesis of *B. ovis* infection in rams was undertaken by Biberstein et al. (1964). In this experiment, 48 rams were artificially infected by conjunctival instillation of an inoculum. Rams were killed in pairs every two to three days during the first two weeks, then weekly for the following two weeks then two-weekly until 255 days after inoculation. The conjunctiva, major lymph nodes, salivary glands, lung, liver spleen, kidney and reproductive organs were analysed histologically and microbiologically. In addition, semen and blood were collected for bacteriological culture and serum was tested for *B. ovis* antibodies using a CFT.

In this trial *B. ovis* was consistently isolated from the site of instillation, the conjunctiva, until a month after infection. From days 11 to 60 the organism could consistently be isolated from lymph nodes. From day 30-45 until the end of the trial on day 255 the organism could be isolated from semen and reproductive organs. At times it was also isolated from the lung, liver, kidney and spleen. At no time was the organism isolated from blood, although blood was only subjected to culture up to two weeks following infection. Histological lesions were seen only in the reproductive tract. These findings

would suggest that following infection onto mucous membranes the organism localises in regional lymph nodes. At some stage during the first month of infection a bacteraemic phase occurs, resulting in localisation of *B. ovis* in the reproductive tract and, at times, other body organs. It appears that *B. ovis* has a predilection for the reproductive tract.

Following infection there is a time delay before significant serological titres occur and before *B. ovis* is shed in the semen. In experimental studies this time delay was variable between rams and between methods of inoculation. Following infection by a "natural" route by inoculation of semen or cell culture onto mucous membranes, it took an average of two to five weeks for development of a significant serological titre (range four days to 11 weeks) and an average of four to nine weeks for *B. ovis* to be shed in semen (range of four to 14 weeks; Table 1.3.1). This suggests that following natural infection there is likely to be a delay of at least two weeks until development of a significant serological titre, and a delay of at least four weeks until the organism is shed in semen.

Table 1.3.1 Time in weeks to seroconversion and positive *B. ovis* semen culture for rams artificially infected with *B. ovis* by inoculation of infected semen or cell culture onto mucous membranes. Rams were sampled weekly after inoculation.

Method of inoculation	**Mean time in weeks (and range) to develop serological titres**	**Mean time in weeks (and range) until positive semen culture**	**Reference**
Semen; nasal	2.3 (0.5-5.5) n=11	n/r (5-10) n=3	Burgess and Norris, (1982)
Semen; penile	5.5 (2-11) n=17	9.5 (5-14) n=6	Laws et al. (1972)
Semen; penile, preputial, rectal or nasal	4.5 (2-9) n=27	7.8 (4-12) n=9	Plant et al. (1986)
Culture; preputial	3.2 (2-5) n=10	4.5 (4-5) n=10	Webb et al. (1980)

Not all rams that are exposed to *B. ovis* shed the organism in semen, although they usually develop serum antibodies. Plant et al. (1986) exposed nine rams to infected semen via the vagina of ewes and inoculated nine rams each with semen either intra-preputially, intra-rectally or intra-nasally. A further five rams were inoculated with *B. ovis* culture into the prepuce. While 36 of the 41 rams developed significant serological titres, only nine shed *B. ovis* in the semen. The remaining 27 rams that did not shed the organism were subsequently slaughtered. There was no microbiological or histopathological evidence of infection in the liver, spleen, kidneys, accessory sex glands, epididymes or regional lymph nodes of these rams. Infections that result in development of serum antibodies but where *B. ovis* is not shed in the semen have been referred to as "transient" infections (Buddle, 1955) or "abortive" infections (Burgess et al., 1982). It can be speculated that these rams are exposed to the organism but are able to overcome the infection before it localises in the reproductive tract (Bailey, 1986).

It would appear likely from the experimental evidence that when a ram becomes persistently infected with *B. ovis*, the organism will localise in at least one if not more of the epididymes, seminal vesicles and ampullae (Biberstein et al., 1964). Thus it is likely that rams that are persistently infected should be identifiable by semen culture.

The long-term persistence of *B. ovis* infection in rams is not well defined. Buddle (1956) reported that a high percentage of infected rams are capable of secreting infected semen for periods in excess of four years, although it was not stated whether the remaining rams discontinued shedding over time. Hartley et al. (1955) reported that some rams could shed *B. ovis* for at least two years. Worthington et al. (1985) reported that three of 42 rams ceased to shed the organism in semen four to 10 months into the course of a 14-month long investigation, but it was unknown how long these rams had been infected prior to the beginning of the investigation. The remaining 39 rams shed *B. ovis* in semen throughout the 14 months. Brown et al. (1973) artificially inoculated 12 rams with *B. ovis* by the conjunctival route, and then subsequently with one or two intravenous injections. One ram stopped excreting the organism in semen after 44 weeks, seven rams stopped shedding after 52 to 70 weeks and four rams continued shedding for at least 91 weeks after which the experiment stopped.

It has been suggested that pre-pubertal rams may have only transient infections and localisation of *B. ovis* in the reproductive tract does not occur (Buddle, 1955). However, infection has been documented in four to six month-old rams (Clapp, 1962; Burgess et al., 1982; Bulgin, 1990a).

1.3.2 Effects on semen quality

Following localisation of *B. ovis* in the epididymes and accessory sex glands of rams, reduced fertility can result due to changes in sperm motility, morphology, concentration and presence of cellular debris.

Sperm motility of affected rams is decreased although there is individual variation between rams. Of 29 naturally infected rams, McGowan and Devine (1960) described 15 as having no sperm motility, seven as having intermediate motility and seven having motility of 60% or greater. Kimberling et al. (1986) undertook semen evaluation of 74 infected and 54 non-infected rams and found significantly lower sperm motility in infected rams ($p<0.05$). A similar result was described by Kott et al. (1988).

During *B. ovis* infection, the characteristic morphological change of sperm is separation of the sperm head from the tail. This finding was first documented in 1942, before the *B. ovis* organism was recognised, following semen evaluation of mixed-age flock rams with epididymitis (Gunn, 1942). Other researchers have since reported a significantly higher number of detached sperm heads in semen from infected rams compared with non-infected rams (Cameron et al., 1971; Cameron and Lauerman, 1976; Kimberling et al., 1986; Kott et al., 1988). Cameron and Lauerman (1976) also reported that a high number of sperm from infected rams had bent tails. Following electron microscopy of sperm from infected rams, Afzal (1985) described detachment and loss of the plasma membrane surrounding the acrosome and suggested that the lesions were due to antibody formed as a result of the infection crossing the epididymal barrier and forming immune complexes with sperm.

Infection has been associated with an increase in inflammatory cells and cellular debris in semen (Jebson et al., 1954; Jebson et al., 1955; Webb et al., 1980). Kimberling et al. (1986) undertook semen evaluation of 74 rams infected with *B. ovis* but without palpable

lesions of epididymitis and found 82% of them to have leukocytes in semen compared with 0% of non-infected rams.

Cameron and Lauerman (1976) reported a significant decrease in sperm output between infected and non-infected rams ($P<0.01$) but there was a large variation in sperm output between rams from both groups and between ejaculates from each ram.

1.3.3 Gross pathology of the reproductive tract

Epididymes

The gross lesions of *B. ovis* infection of the epididymis have been described in detail by Jebson et al. (1955) and Kennedy et al. (1956), but details of the number of specimens examined are not given in either report. Epididymal lesions are usually characterised by enlargement of the tail of one or both of the epididymes. This may vary from a slight enlargement to a four to five-fold increase. It was reported that in approximately 90% of rams, lesions were confined to the tail of the epididymis (Kennedy et al., 1956). On cutting into the epididymal tissue, there is an increase in intraepididymal connective tissue and thickening of the overlying albuginea. In some cases the epididymes contain solitary or multiple sperm granulomas with contents ranging from creamy-tan fluid to inspissated, caseous material. Haemorrhage and exudative inflammation in the tunica vaginalis is a frequent finding - in more chronic cases this results in adhesions between the two layers of the tunica vaginalis (Blasco, 1990). Adhesions between the tail of the epididymis, the parietal tunica vaginalis and the distal pole of the testicle are common.

Shott and Young (1971) attempted to investigate whether the inflammatory reaction in the epididymes resulted from an inflammatory reaction to the bacteria itself or from extravasation of sperm into the interstitium inciting a foreign-body reaction, but the results were inconclusive. Paolicchi et al. (2000) demonstrated that *B. ovis* infection in rams is associated with an anti-spermatic response but there was no discussion on the relative contribution of this response to the pathological lesions.

Not all infected rams develop epididymitis. Of 88 commercial rams excreting *B. ovis* in semen, 30 (34%) had palpable lesions of epididymitis whereas no lesions were detected in the remaining 58 rams (Hughes and Claxton, 1968).

Accessory sex glands

While *B. ovis* localises and causes histological lesions in the seminal vesicles and ampullae of infected rams, gross lesions of these organs were not seen by Kennedy et al. (1956) or Foster et al. (1987), although Jebson et al. (1955) reported that in a few cases the seminal vesicles were larger and of firmer texture than normal. Blasco (1990) described frequent enlargement of the seminal vesicles with dilated or fluid-filled ducts observed on the cut surface. This latter observation was published in a review of ovine brucellosis and is not referenced. There were no details on numbers of rams examined or whether organs from control rams were examined concurrently.

Other organs

There are only a few reports of lesions associated with *B. ovis* infection in organs other than the epididymes and accessory sex glands. Secondary testicular atrophy on the affected side or sides may occur (Kennedy et al., 1956). The inguinal and iliac lymph nodes may be enlarged and oedematous (Simmons and Hall, 1953; Jebson et al., 1955). Gross lesions of other body organs from infected rams have not been described.

1.3.4 Histopathology of the reproductive tract

Epididymes

The histological lesions in the reproductive tract of rams infected with *B. ovis* have been described by Kennedy et al. (1956), Biberstein et al. (1964) and Rahaley and Dennis (1984). Early histological lesions in the epididymes are characterised by perivascular oedema and inflammatory cell infiltration into the interstitium. Lymphocytes tend to predominate, although macrophages and neutrophils are also present. Lymphatics are often dilated and packed with inflammatory cells and there may be hyperplasia of vascular endothelium. Epididymal ducts may be filled with inflammatory cells and necrotic debris and there is hyperplasia of the epididymal epithelium and intraepithelial cyst formation. The epithelial changes and narrowing of the ducts lead to extravasation of sperm into the

interstitium causing further interstitial inflammation and eventually fibrosis. Extravasation of sperm may result in the formation of sperm granulomas which are characterised by a central core surrounded by a rim of epithelioid and giant cells and then a rim of active fibrosis. Similar epididymal lesions were seen in gerbils and white-tailed deer artificially inoculated with *B. ovis* (Cuba-Caparo and Myers, 1973; Barron et al., 1985).

Accessory sex glands

Histological lesions of the accessory sex glands of rams were seen only in the seminal vesicles and ampullae, characterised by infiltration of the lamina propria and epithelium by plasma cells, macrophages, lymphocytes and neutrophils. Lymphoid follicles may also be present (Biberstein et al., 1964; Foster et al., 1987).

Other organs

Lesions in the testicles are reported to be rare and appear to be confined to testes that have undergone atrophy due to extensive lesions in the epididymes. In these cases the testes contained foci of sperm stasis, intratubular granulomas and calcification with surrounding fibrosis (Kennedy et al., 1956). Biberstein et al. (1964) were able to isolate *B. ovis* from the lymph nodes, salivary glands, lung, liver, spleen and kidney of infected rams but found no histopathological lesions in any of these organs.

1.3.5 Microbiological isolation

Due to its growth characteristics, *B. ovis* can be difficult to isolate. The organism requires culture media enriched with blood or serum incubated in a 10% CO_2 atmosphere (Burgess, 1982). After 48 hours incubation, colonies are just visible and after five days incubation they are approximately two millimeters in diameter (Buddle and Boyes, 1953). The slow-growth of *B. ovis* means that in the presence of contamination with more rapidly-growing organisms such as *Proteus* or fungi, colonies of *B. ovis* can either be overgrown or difficult to visualise. For this reason a selective media is commonly used, containing the antibiotics vancomycin, colistimethate, nystatin and furadantin (Brown, 1971).

The organism can be isolated from the semen of infected rams but it has been suggested that shedding of *B. ovis* in semen may be intermittent in some rams (Hughes and Claxton,

1968; Worthington et al., 1985). However, this observation has not been well defined and it is possible that at times *B. ovis* is not isolated due to overgrowth of contaminating organisms (Biberstein and McGowan, 1958). Alternatively it is possible that in some rams the organism is only present in one part of the reproductive tract, for example the epididymes, and therefore *B. ovis* would not be isolated from ejaculates that do not contain an epididymal fraction.

At necropsy, *B. ovis* has been isolated from the seminal vesicles, ampullae, bulbo-urethral glands and one or both epididymes from the majority of infected rams, and from the testes of some rams (Table 1.3.2). In some infected rams the organism was isolated from lymph nodes, spleen, kidney, liver and lung (Table 1.3.3).

Table 1.3.2 Number (and percentage) of bacteriological isolations of *B. ovis* from reproductive organs of rams variable times after establishment of infection.

Number of rams	Epididymes	Seminal vesicles	Ampullae	Bulbo-urethral	Testes	Reference
30†	25 (83)	23 (77)	25 (83)	14 (47)	14 (47)	Biberstein et al. (1964)
6	3 (50)	6 (100)	6 (100)	n/r	1 (17)	Plant et al. (1986)
49	42 (86)	28 (57)	41 (84)	4 (8)	6 (12)	Searson (1986)
12	7 (58)	9 (75)	6 (50)	n/r	6 (50)	Shott and Young (1971)
11	8 (73)	7 (64)	8 (73)	7 (64)	n/r	Worthington et al. (1985)

n/r: not reported

†: number only includes rams which were shedding *B. ovis* in semen

Table 1.3.3 Number (and percentage) of bacteriological isolations of *B. ovis* from body organs of rams variable times after establishment of infection.

Number of rams	Regional lymph nodes	Spleen	Kidney	Liver	Lung	Reference
30†	13 (43)	7 (23)	14 (47)	7 (23)	2 (7)	Biberstein et al. (1964)
6	1 (17)	1 (17)	2 (33)	0	n/r	Plant et al. (1986)
12	n/r	2 (17)	3 (25)	n/r	n/r	Shott and Young (1971)
11	1 (9)	1 (9)	1 (9)	n/r	2 (18)	Worthington et al. (1985)

n/r: not reported

†: number only includes rams which were shedding *B. ovis* in semen

1.4 Pathophysiology of *B. ovis* infection in ewes

1.4.1 Pathogenesis

The pathogenesis of *B. ovis* infection in ewes was investigated by Muhammed et al. (1975), by inoculating *B. ovis* onto the vagina of 20 ewes in early pregnancy. Starting from the fourth week after infection, ewes were killed at random at two to four week intervals for bacteriological examination of uterus, foetus, foetal membranes, udder, lymph nodes, vagina and blood. Nine ewes were kept alive until parturition. In ewes killed 29 days after inoculation, *B. ovis* was isolated only from the mammary and inguinal lymph nodes. It was isolated from the blood of nine ewes from 22 days and up to 91 days after inoculation, and from the vagina of five ewes for up to 64 days after inoculation. *Brucella ovis* was not isolated from the uterus, foetus or foetal membranes of any of the ewes. The number of ewes used in this trial was small but it appears that following inoculation of *B. ovis* into the vagina, the organism localises in the regional lymph nodes for a short period of time before a bacteraemia occurs. It would appear that the organism can persist in the vagina of some ewes for at least two months.

The pathogenesis of infection in the pregnant ewe was investigated by Hartley (1961), who examined placentae and foetuses from both naturally occurring and experimental cases of *B. ovis* abortion. He suggested that during the bacteraemic phase in pregnant ewes the organism reaches the uterus and localises in the chorionic epithelium, causing necrosis of these cells. This necrosis leads to an extensive inflammatory reaction in the underlying mesenchyme along with severe vasculitis which in turn leads to ischaemic necrosis of the chorio-allantois and associated cotyledons. At some stage the organism also invades the foetal cotyledons, resulting in necrosis of the foetal villi. Late in the infection *B. ovis* enters the foetus, possibly taken in with contaminated amniotic fluids. With extensive placental lesions the lambs may die *in-utero* and abortion results, usually in the last trimester of pregnancy. Alternatively the lambs may be born alive and either die soon after birth or remain viable.

1.4.2 Effects on reproductive performance

Brucella ovis infection of ewes has been reported to result in late-term abortions or birth of weak lambs that die soon after birth (Lawrence, 1961). However, while the pathology, histopathology and microbiology of *B. ovis* infection in ewes have been well documented, in many cases ewes were infected during pregnancy, commonly by intravenous injection of *B. ovis* organisms (Table 1.4.1). Clearly this method of infection does not mimic "natural" infection in which ewes are likely to become infected by mating with infected rams. In the three experiments where ewes were infected by a more natural route, either by mating to infected rams or by intravaginal inoculation, effects were relatively minor (Table 1.4.1), (Buddle, 1955; Hartley et al, 1955; Hughes, 1972a).

Table 1.4.1 Experimental infection of ewes at various stages of pregnancy with *B. ovis* by a variety of routes, and success of infection measured by development of a serological titre and pathological or microbiological evidence of infection in the foetus or placenta.

Time of infection	Route of infection	Dose (colony forming units)	Number of ewes	Number developing a persistent CFT titre	Pathological or microbiological evidence of infection in the foetus or placenta	Reference
Pre-mating	Subcutaneous	$1.5x10^{10}$	12	n/r	2 (17%)	Buddle (1954)
	Conjunctival	$1x10^{9}$	5	0	0	Osburn and Kennedy (1966)
	Intrauterine	$1x10^{9}$	4	0	0	Osburn and Kennedy (1966)
Day of mating	Mating to infected ram	-	25	n/r	2	Buddle (1955)
	Mating to infected ram	-	21	n/r	0	Hartley et al. (1955)
	Intravaginal	$2.8x10^{9}$	46	19 (41%)	3† (7%)	Hughes (1972a)
	Intravenous	$2.8x10^{9}$	33	33 (100%)	15§ (45%)	Hughes (1972a)
First trimester of pregnancy	Intravenous	$1x108\text{-}10^{9}$	115	n/r	64 (56%)	Hartley et al. (1954)
	Oral	$2.5x10^{10}$	9	n/r	5 (56%)	Buddle (1955)
	Conjunctival	$1x10^{9}$	3	2 (67%)	2 (67%)	Osburn and Kennedy (1966)
	Intrauterine	$1x10^{9}$	3	0	0	Osburn and Kennedy (1966)
	Intravenous	$5.4x10^{7}$	36	n/r	27 (75%)	Ris (1970)
	Intravenous	$2.8x10^{9}$	38	38 (100%)	24 (63%)	Hughes (1972a)
	Intravaginal	$3.5x10^{7}$	20	16 (80%)	0	Muhammed et al. (1975)
Second trimester of pregnancy	Intrauterine	$1x10^{9}$	9	9 (100%)	9 (100%)	Osburn and Kennedy (1966)
	Conjunctival	$1x10^{9}$	10	8 (80%)	8 (80%)	Osburn and Kennedy (1966)
	Conjunctival	$1.3x10^{9}$	24	45 (100%)	3 (13%)	Grillo et al. (1999)
Third trimester of pregnancy	Intravenous	$6x10^{9}$	12	n/r	1 (8%)	Collier and Molello (1964)
	Conjunctival	$1x10^{9}$	2	0	0	Osburn and Kennedy (1966)
	Intrauterine	$1x10^{9}$	4	4 (100%)	3 (75%)	Osburn and Kennedy (1966)
	Intravenous	n/r	9	9 (100%)	3 (33%)	Meinershagen et al. (1974)
	Oral	n/r	10	6 (60%)	0	Meinershagen et al. (1974)
	Conjunctival	$1.3x10^{9}$	16	16 (100%)	0	Grillo et al. (1999)

n/r: not reported

†: All three lambs remained viable. Placentae only were infected.

§: 12 of the 15 lambs remained viable. From these, placentae were infected

There are few field reports of *B. ovis*-induced abortion in commercial ewe flocks. In New Zealand, *B. ovis* was incriminated in causing late-term abortion in 50 ewes from a flock of 1100 (McFarlane et al., 1952) although it has been reported retrospectively that Toxoplasmosis may also have been present in this flock (Hughes, 1972a). In Victoria, Australia, Gorrie (1962) suggested that abortion of ewes due to *B. ovis* was so rare it was of curiosity value only. In one report from the United States of America, an abortion storm with 150 of 300 ewes aborting was attributed to *B. ovis* (Libal and Kirkbride, 1983) but in this study only three foetuses were submitted for laboratory analysis with *B. ovis* isolated from two. In South Australia, *B. ovis* infection was incriminated in causing a 20 to 30 percent decrease in lambing percentage due to late abortions and neo-natal death on properties with a high prevalence of infection in rams (Keogh et al., 1958) but objective data such as bacterial isolation from dead lambs was not reported.

Hughes (1972a) suggested that sporadic deaths of at-term lambs may be a more common manifestation of *B. ovis* infection than abortion and speculated that at-term lambs could be mistaken for aborted foetuses based on birth-weight. He artificially infected 46 ewes by vaginal inoculation (Group A) and 50 ewes by intravenous injection (Group B) on the day of mating. A further 46 were infected by intravenous injection 10 to 14 days after mating (Group C) and 93 control ewes were used. There was no significant difference in gestation length between groups of ewes but lambs born to Group C ewes had a significantly reduced birth weight compared with controls. Rickard-Bell (1963) described perinatal lamb mortality rates of one to two percent attributed to *B. ovis* infection in an intensively managed New South Wales Merino flock. Haughey et al. (1968) examined lamb carcasses from 8 flocks where *B. ovis* infection was present in rams and concluded perinatal loss due to *B. ovis* infection affected an average of 0.9% of lambs.

During experimental studies with intensive monitoring at lambing it was found that some lambs may be born with infected placentae but remain viable. Hartley et al. (1954) artificially inoculated 101 ewes intravenously at one to three weeks gestation. Twenty-four of the ewes had *B. ovis* cultured from the placentae but produced live lambs. Hughes (1972b) artificially infected 142 ewes either by intravaginal or

intravenous inoculation on the day of mating or intravenous inoculation at 10-14 days gestation. *Brucella ovis* was isolated from the placenta of 42 ewes at parturition but 29 of these ewes gave birth to live lambs.

Compared with non-infected ewes, a significantly higher proportion of ewes intravenously inoculated with *B. ovis* on the day of mating failed to hold to service (Hughes, 1972a). This suggests that a bacteraemia at the time of mating may result in a reduced conception rate.

Serological evidence of infection in commercial flocks

In commercial ewe flocks mated to *B. ovis* infected rams, the seroprevalence has ranged from five to 19% (Watts, 1955; Wellington, 1955; Marco et al., 1994). Snowden (1958) found 92 of 260 ewes (35%) in one stud Southdown flock in Australia to be seropositive. He tested these ewes annually for three years and found that around 60% of previously seropositive ewes became seronegative per year, suggesting that ewes do not maintain antibodies for long periods of time. Keogh et al. (1958) mated a ewe flock containing between 300 to 320 ewes to infected and non-infected rams. Sixteen to 24 ewes (5-8%) became seropositive per year. While the presence of serum antibody appears to be transient in most ewes, in this study eight ewes remained seropositive for three years.

Carry over of infection into the second year

Snowden (1958) reported that only a small proportion of ewes maintain serum antibody titres for any length of time. Similarly, it would appear to be uncommon for ewes to show systemic signs of infection (abortion) for two years in a row. Of 26 ewes that had diseased placentae at the end of their first pregnancy, only three (12%) had diseased placentae at the end of their subsequent pregnancy (Buddle, 1955). Fourteen of 24 ewes experimentally infected at days 45-75 or 120 of pregnancy remained seropositive until the end of their second pregnancy. None of these aborted, shed *B. ovis* in vaginal discharges or had *B. ovis* isolated from their lambs but three shed the organism in milk. Sixteen of the 28 lambs born were seropositive (Grillo et al., 1999).

Congenital infection in lambs

Serum antibodies against *B. ovis* infection have been found to be present in some young lambs. Grillo et al. (1999) artificially infected 40 ewes in mid to late pregnancy by conjunctival inoculation. At lambing and during lactation around 60% of the ewes were actively excreting *B. ovis* in milk and when sampled at two weeks of age, 27 of the 46 surviving lambs had serum antibodies. Serum antibodies persisted in these lambs for an average of 37 days (maximum 117 days). From experimental studies where ewes were artificially infected with *B. ovis* during pregnancy, it has been demonstrated that the foetal lamb is able to produce *B. ovis* antibodies in response to infection (Biberstein et al., 1966; Osburn and Kennedy, 1966; Tierney et al., 1997). Ris (1970) demonstrated *B. ovis* complement fixation antibody titres in the colostrum of infected ewes, and found that the colostral titres were higher than titres in serum collected from the ewes at the same time. Thus it would seem probable that the presence of serum antibody in lambs may be due either to active immunity from exposure to infection *in utero* or exposure from ingesting infected milk, or passive immunity from ingestion of colostral antibody.

It is not clear if ram lambs born to infected ewes may themselves become infected and develop clinical *B. ovis* infection. Hughes (1972b) was able to culture *B. ovis* from the testes of ram lambs that had been born dead from infected ewes. For a nine month period after weaning Buddle (1955), monitored seven ram lambs born from experimentally infected ewes that had grossly diseased placentae. None of these rams developed epididymitis over this time but serology and semen culture were not attempted. Bulgin (1990a) suggested that congenital infection may have contributed to a *B. ovis* epizootic in virgin ram hoggets but the possibility of infection originating from another source could not be ruled out. Rickard-Bell (1963) reported that ram lambs born to ewes that had been mated by infected rams had a five percent incidence of *B. ovis* at 14 months of age. He suggested that infection may have been contracted in the maternal environment but again, the possibility that infection may have come from a straying infected ram could not be excluded.

1.4.3 Gross pathology

McFarlane et al. (1952), Hartley (1961) and Osburn and Kennedy (1966) have described the pathology of foetal membranes from ewes that abort due to *B. ovis* infection. The main feature is gross, gelatinous oedema of the chorio-allantois with a thickened amnion which is adherent to the chorio-allantois. Diseased cotyledons vary in appearance and changes include colour changes, increased firmness and gross enlargement. The intercotyledonary areas have elevated yellowish-white-grey thickenings or plaques which may coalesce to give large areas with the appearance of chamois leather. In mild cases, there may be a yellow mucopurulent material adhering to the chorionic surface of the chorio-allantois. In some cases this exudate has been described as red-brown in colour with small yellow flecks. One horn may be more affected than the other.

Van Rensburg et al. (1958) described a mild vagino-cervicitis in ewes served by rams infected with *B. ovis*. It was reported that this subsided within a day or two but in ewes that did not conceive it sometimes reappeared at the next oestrus. In ewes that did conceive and lamb normally it was observed to recur after parturition. Mucopurulent exudate was present in the uterus of five ewes slaughtered soon after abortion and there was oedema of the broad ligament of one ewe but no other gross abnormalities were noted (Hartley, 1961).

Hartley (1961) and Osburn and Kennedy (1966) have described the pathological features of aborted foetuses. Grossly, aborted foetuses show variable pathology depending on the stage of pregnancy at which abortion occurred and the level of autolysis. Mild to moderate clear or bloody subcutaneous oedema is present along with mild to moderate excesses of clear or bloody fluid in body cavities, often with strands or flecks of fibrin. A "salt-like" or "chalk-like" deposit of white material on the walls and/or soles of the hooves and accessory digits of approximately 50% of aborted lambs has been described. These deposits disappeared when the hooves were immersed in water. These deposits were also described by Meinershagen et al. (1974).

1.4.4 Histopathology

The histopathology of the foetal membranes and foetus have been described in detail by Hartley (1961) and Osburn and Kennedy (1966). In the placenta, the major lesions reported were extensive inflammatory and necrotic changes. There were marked changes in the placental vasculature consisting of fibrinous thrombi in the lumen of vessels, inflammatory cell infiltration of the adventitia, fibrinous depositions in the intima and perivascular fibrosis. Cotyledonary changes involved oedema or necrosis of the foetal villi with accumulations of neutrophils at the base with large numbers of *B. ovis* organisms in the trophoblastic epithelium. Where the amnion was adherent to the chorio-allantois, Hartley (1961) described oedema, haemorrhage and fibroblastic and capillary proliferation of the amnion.

Histopathological examination of the uteri of five ewes that aborted due to *B. ovis* infection showed minor increases in mononuclear cell numbers in the subepithelial stroma. Neutrophils containing *B. ovis* were present in the lumen of some uterine glands located at the periphery of the plancentome (Hartley, 1961).

In aborted foetuses, the foetal lung showed lesions of peribronchiolar lymphoreticular nodules with occasional macrophage and neutrophil accumulation in the peribronchial lymphatics. Alveolar septal swelling was also noted (Osburn and Kennedy, 1966). These authors also described lymphadenitis in the bronchiolar and mediastinal lymph nodes, increased cellularity in the cords of the red pulp of the spleen, large numbers of neutrophils in the portal areas of the livers of some foetuses and mononuclear infiltrates in the portal areas of the liver of others, and renal lesions in some foetuses consisting of a diffuse neutrophilic and oedematous infiltrate at the corticomedulllary junction.

1.4.5 Microbiological isolation

Where *B. ovis* infection in ewes has resulted in abortion or grossly visible diseased placentae, the organism can consistently be recovered from the placenta (Collier and Molello, 1964; Osburn and Kennedy, 1966). In foetuses from which diseased foetal membranes were present, *B. ovis* could be isolated with varying success from the lungs,

abomasal contents and spleen (Hartley et al., 1954; Collier and Molello, 1964; Osburn and Kennedy, 1966; Ris, 1970; Libal and Kirkbride, 1983).

Brucella ovis was isolated from the blood of intra-vaginally inoculated ewes beginning 22 days after infection and ending 98 days after infection (Muhammed et al., 1975). The organism was isolated at parturition, during lactation and at weaning from the milk of 45 to 65% of ewes infected by the intravenous route during mid pregnancy (Grillo et al., 1999).

Muhammed et al. (1975) reported that *B. ovis* was isolated up to 64 days after inoculation from the vagina of ewes experimentally infected by the vaginal route. Van Rensburg et al. (1958) inseminated an oestrus ewe with semen from an experimentally infected ram. At the next oestrus period 17 days later, vaginal mucus from this ewe was applied to the glans penis of a non-infected ram which subsequently developed infection, suggesting *B. ovis* can remain viable in vaginal mucus for at least 17 days. Hartley et al. (1954) reported that vaginal discharges collected from ewes up to two weeks post-abortion contained *B. ovis*. In ewes receiving an intra-venous inoculation of *B. ovis* in mid-preganancy, Grillo et al. (1999) reported that four weeks after inoculation the organism was isolated from the vagina of four of 24 ewes. At lambing the organism was isolated from the vagina of 20 of the 24 ewes and at weaning *B. ovis* could still be isolated from the vagina of eight. Only two of the 24 ewes gave birth to still-born lambs from which *B. ovis* was isolated.

Of naturally and experimentally infected non-pregnant ewes *B. ovis* was isolated with varying success from the uterus, mammary gland, spleen and the iliac, cranial, scapular, prefemoral and mammary lymph nodes (Marco et al., 1994; Grillo et al., 1999). In some of the ewes infection was extra-uterine only.

1.5 Serodiagnosis of *B. ovis* infection in sheep

1.5.1 Introduction - diagnosis of *B. ovis* in sheep

In live rams the methods used for the diagnosis of *B. ovis* include detection of lesions of epididymitis by scrotal palpation, culture of semen and serological tests (West and Bruce, 1991).

In rams, the presence of epididymitis can be suggestive of, but is not specific for, *B. ovis* infection (Edgar, 1959). Epididymitis is also caused by Gram-negative pleomorphic organisms (*Actinobacillus seminis/Histophilus ovis*) or, rarely, by other bacterial organisms (Bulgin, 1990b). While there are some age and sexual experience-related patterns in the incidence of either *B. ovis* or Gram negative pleomorphic infection in rams (Bulgin and Anderson, 1983) these are by no means absolute (Burgess et al., 1982). As discussed in Chapter 1.3.3, not all rams that are infected with *B. ovis* develop epididymitis. Thus scrotal palpation in conjunction with the history of a flock may give an indication of the infection status of a flock but could not be considered an accurate diagnostic indicator, particularly on an individual ram basis (Hughes and Claxton, 1968). Eradication of *B. ovis* based on culling of rams with epididymitis was unsuccessful (Clapp et al., 1955; Bagley et al., 1985).

Bacteriological culture of semen is a useful diagnostic test of infection in rams, as a positive *B. ovis* culture is considered definitive evidence of infection (Brown, 1971). This is a useful test in doubtful cases of *B. ovis* infection because it may detect infected rams that would otherwise not have been detected (West and Bruce, 1991). However, collection of semen from rams for *B. ovis* culture requires skill and specialised equipment, and bacteriological culture of semen is relatively expensive. There has also been a suggestion that in some rams shedding of *B. ovis* in semen is intermittent (Worthington et al., 1985) although this has not been well defined.

Thus in live sheep, for flock and individual animal diagnosis of *B. ovis* infection, serology is considered to be the most practical diagnostic test (Hughes and Claxton, 1968).

1.5.2 Use and interpretation of serological tests

Discussion of the interpretation of diagnostic tests can be found in most epidemiological texts, for example Thrusfield (1995) and Noordhuizen et al. (1997).

Some tests are dichotomous, in that the result is either positive or negative (for example, isolation of *B. ovis* from semen), but for the majority of serological tests the results are on a continuous scale and a "cut-off" value must be determined in order to condense the result into a dichotomous scale (diseased or non-diseased). However, any cut-off value using a continuous scale is likely to result in an overlap between the diseased and non-diseased populations (Figure 1.5.1). In the case of *B. ovis*, culture of the organism from either semen or the reproductive tract is an accurate indication of infection and is therefore regarded as the "gold standard". The characteristics of the serological test relative to the gold standard are defined as sensitivity and specificity.

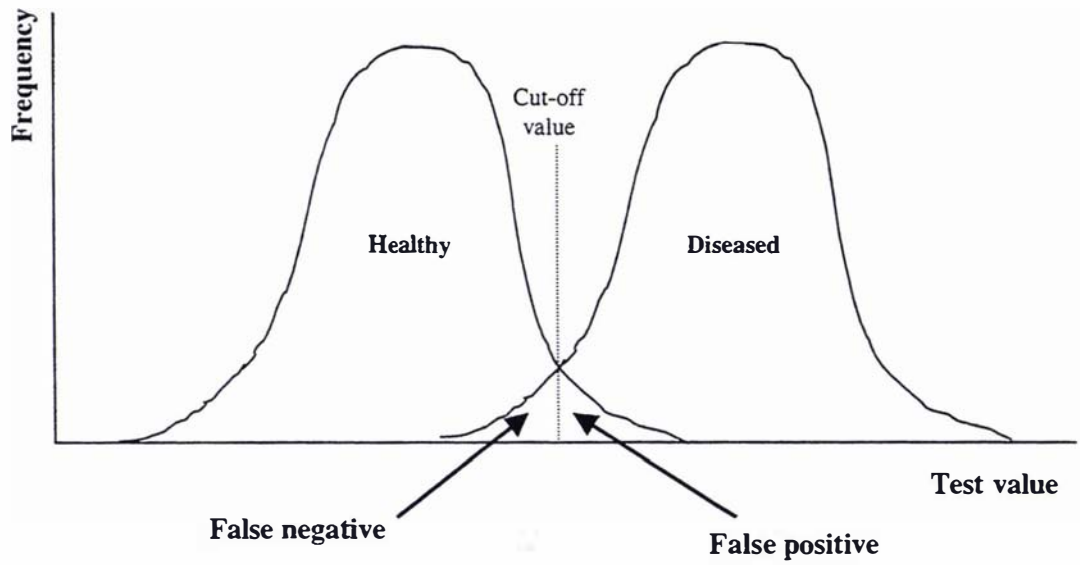


Figure 1.5.1 Test result measured on a continuous scale showing a bimodal normal distribution of values for healthy and diseased animals, with an overlap in the middle where false-positive and false-negative results may occur.

Sensitivity and specificity

The sensitivity of a test is the ability of the test to correctly identify a diseased animal and is defined as the proportion of animals with the disease that test positive. Specificity represents the ability of the test to correctly identify non-diseased animals and is defined as the proportion of animals without the disease that test negative. For serological tests where results are measured on a continuous scale, the decision on where to place cut-off values will influence the sensitivity and specificity. Ideally, cut-off values should be chosen according to the objective of testing. For example, in a flock known to be infected with *B. ovis* the objective is to correctly identify all diseased animals and therefore a cut-off value should be chosen to give maximum sensitivity, even though specificity may be compromised (sera from some animals may give false-positive results). Alternatively, in a flock that is free from *B. ovis* with no history of possible introduction, a cut-off value to give maximum specificity may be chosen to minimise false-positive results (Reichel et al., 1999). In reality, cut-off values tend to be

chosen to give an optimum balance between sensitivity and specificity and these values are then applied to all flocks, regardless of their infection status.

Predictive values

Predictive values quantify the probability that a test result for a particular animal correctly identifies the condition. The positive predictive value is the proportion of test-positive animals that truly have the disease and the negative predictive value is the proportion of test-negative animals that do not have the disease. These values are calculated using the sensitivity and specificity of the test, but they also depend on the prevalence of the disease in the population. For example, if the disease prevalence in the population is very low then the positive predictive value will be decreased.

Use of serological tests

None of the serological tests used for the diagnosis of *B. ovis* have 100% sensitivity or specificity and there is always the opportunity for false-positive or false-negative interpretations to occur. Therefore the interpretation of serological tests for *B. ovis* is not always simple and it is important that results are considered in conjunction with flock history and clinical signs (Worthington, 1982a). Where results are contradictory, other diagnostic procedures such as the use of several serological tests in parallel or semen culture should be employed (West et al., 1993; Reichel et al., 1994).

1.5.3 The Complement Fixation Test (CFT)

Complement fixation tests involve inactivating the inherent complement within the serum of the animal to be tested, making serial dilutions and then adding an appropriate amount of antigen, a known amount of guinea pig complement and sheep red blood cells. If antibody is present in the serum, it binds to the antigen and the complement is fixed, thus preventing lysis of the red blood cells.

The initial *B. ovis* CFT was developed by Clapp et al. (1955) using a whole-cell antigen emulsified in guinea-pig serum. This method was used to test serum from 126 rams with good results (Clapp, 1955). In an effort to reduce the degree of anticomplementary activity with this test, Biberstein and McGowan (1958) and Clapp (1961) experimented

with various methods of antigen preparation. It was concluded that use of cell-free antigen resulted in less anti-complementary activity and an enhanced sensitivity.

The original CFT was described as a "warm" CFT, as fixation of complement occurred by incubation at 37°C for 30 minutes. Development of a "cold" CFT in which fixation of complement was allowed to take place for 18 hours at 4°C was first described by Ris (1967). A comparison between the warm and cold complement fixation tests concluded that the cold test was slightly more sensitive than the warm test (Ris, 1974; Burgess and Norris, 1982) but that it resulted in a small increase in anti-complementary activity (Ris, 1974). Further work comparing antigen preparation and complement fixation method confirmed the superiority of cell-free antigen in a cold CFT (Anon, 1983). A microtitre CFT method was described by Searson (1982), and Weddell (1974) reported on an automated CFT.

In New Zealand, the CFT is the main test used for screening and diagnosis of *B. ovis* infection in rams (Reichel and West, 1997). The test is a cold, non-automated microtitre CFT (G Ross, *pers. comm.*) which is performed at doubling dilutions from 1:4 to 1:128. Using cut-offs where reactions from 1/8 to 3/8 are considered suspicious, with lower titres considered negative and higher titres considered positive, it has a reported sensitivity and specificity of 96.3% and 99.3% respectively (Worthington et al., 1984). While there have been concerns from veterinary practitioners about false-positive and false-negative reactors in this test (Hicks et al., 1978; Hughes, 1982; Wagner, 1982), this is an inevitable finding with virtually any diagnostic test. The *B. ovis* CFT has proven an effective tool for eradication of brucellosis in flocks (Bruere, 1982).

Problems associated with the CFT for diagnosis of *B. ovis* in rams include anti-complementary reactions from the serum of some rams, particularly when the samples are contaminated or haemolysed (Worthington and Cordes, 1981) and fluctuating low positive-suspicious-negative titres in some rams (Webb et al., 1980). It was suggested that false-negative reactions may occur in some rams with chronic infections (Worthington, 1982b) although in later work no evidence was found to suggest that the tendency to react negatively to the CFT increased over time (Worthington et al., 1985).

1.5.4 The Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbant assay (ELISA) tests are based on coating a well with antigen and incubating this with serum from the animal to be tested, and an antibody-enzyme conjugate. If antibody is present in the serum, it binds to the antigen allowing the antibody-enzyme conjugate to binds. Addition of a substrate results in a colour change or flouresence. ELISA results are measured by the absorbance of the substrate solution. The log of this absorbance is directly proportional to the log of the antibody concentration and so ELISA titres are calculated and reported as antibody units per ml (Worthington et al., 1984).

A number of authors have described ELISA tests for use in the diagnosis of *B. ovis* infection (Ris et al., 1984; Spencer and Burgess, 1984; Lee et al., 1985; Walker et al., 1985; Cho and Niilo, 1987). As with the CFT, heat-extracted cell-free antigen is generally used although a whole cell ELISA test has been reported (Chin et al., 1991).

There is general agreement that the sensitivity of the ELISA is marginally better than that of the CFT while still retaining a high degree of specificity (Rahaley et al., 1983; Spencer and Burgess, 1984; Worthington et al., 1984; Lee et al., 1985; Cho and Niilo, 1987; Marin et al., 1989) although in some cases relatively small numbers of rams were tested. The ELISA has the ability to detect all classes of antibody (Spencer and Burgess, 1984) whereas the CFT is able to detect IgG_1 antibody but not IgG_2 antibody, because ovine IgG_2 does not fix guinea-pig complement (Cho and Niilo, 1987). It has been suggested that these factors may help explain the slight differences between the performance of the CFT and ELISA.

In New Zealand an automated ELISA test is used (G Ross, *pers. comm.*). The current cut-off values for use in sheep are between 75 and 150 for suspicious reactions. Titres lower than 75 are considered negative and titres greater than 150 are considered positive. Worthington et al. (1984) reported a sensitivity and specificity of 97.2% and 98.6% respectively using cut-off values of 400 and 500 for suspicious reactions. Reichel et al. (1999) found similar results using cut-off values of 114 and 133 for suspicious results. He suggested that different cut-off values be adopted for each flock

depending on the flock history. The *B. ovis* ELISA test is not routinely used in New Zealand, but in situations where CFT results are conflicting it may be used in parallel with the CFT to enhance overall sensitivity (West and Bruce, 1991; West, 2000).

Advantages of the ELISA test include a slightly increased sensitivity over the CFT, no anti-complementary activity as is sometimes seen with the CFT, no requirement for serum inactivation, ease of automation and the requirement for only one serum dilution (Worthington et al., 1984). Disadvantages are that the ELISA requires an expensive plate meter, calculation of results is more complex than the CFT and it has a slightly lower specificity than the CFT (Worthington et al., 1984). Interpretation of results with low reactions can be difficult (Hilbink et al., 1993). Overall, the ELISA is likely to be as useful as the CFT for large-scale testing of flocks.

1.5.5 The Gel Diffusion Test (GDT)

Matthews and Trueblood (1967), demonstrated that *B. ovis* would cause precipitating antibodies to form and developed an effective gel-diffusion test for detection of these antibodies. Myers and Siniuk (1970) reported on a simple gel-diffusion test for detection of *B. ovis* antibodies and later evaluated it in the field where it compared favourably with a CFT (Myers, 1973). In New Zealand the gel diffusion test is performed in a petri dish using a seven-well pattern. This test has a reported sensitivity and specificity of 91.7% and 100% respectively (Worthington et al., 1984). While not routinely used in New Zealand, the high specificity of this test makes it the test of choice for confirmation that suspected false-positive reactors in the CFT or ELISA are indeed false positives (Hilbink et al., 1993).

1.5.6 Other tests

Skin sensitivity

Hall (1955) described a skin test where *B. ovis* antigen was injected intradermally and the test read three to four days later. This test has not been referred to since, presumably because it was superseded by more sophisticated tests such as the CFT.

Haemagglutination

Ris and Te Punga (1963) developed an indirect haemagglutination test for the detection of *B. ovis* antibodies in sera and later evaluated it on 25 infected and non-infected rams in comparison with scrotal palpation, semen culture and CFT (Ris, 1964). The performance of the haemagglutination test was similar to the CFT but this test does not appear to have been further used.

Immunofluoresence

Cox et al. (1977) described a simple immunofluoresence test to detect *B. ovis* antibody that was tested on sera from 54 infected and non-infected rams. It gave comparable results to a CFT and was more sensitive than a GDT. Ajai et al. (1980) used immunofluoresence to directly identify *B. ovis* organisms in cultural smears.

Counterimmunoelectrophoresis

Myers et al. (1972) isolated an R antigen from the surface of *B. ovis* organisms and used this antigen in a counterimmunoelectrophoresis test to detect *B. ovis* antibody in the sera of infected and non-infected rams (Myers, 1979). The test performed favourably when compared with a CFT and a GDT.

Electrophoretic Immunoblotting (EIB)

Electrophoretic immunoblotting techniques for detection of *B. ovis* antibodies have been described by Chin et al., (1991), Kittelberger et al. (1994) and Kittelberger et al. (1997). It was concluded by Chin et al. (1991) that overall, immunoblotting offered no advantage over CFT or ELISA as a primary assay for *B. ovis*. When used to test infected and non-infected ram and deer sera, Kittelberger and Reichel (1998) found a sensitivity and specificity in rams of 98.6% and 99.1% respectively and in deer of 94.4%

and 100% respectively. In New Zealand the EIB technique is not routinely used as a diagnostic test.

1.6 Prevalence and economic importance of *B. ovis* infection in sheep

It is difficult to estimate the prevalence of *B. ovis* infection in rams worldwide but historically in Australia, prior to the classification of *B. ovis*, it was reported that during routine palpation of commercial flock rams for breeding soundness large numbers had lesions of epididymitis. The incidence of epididymitis in rams in Queensland and New South Wales was estimated to be between five and six percent (Gunn, 1942; Miller and Moule, 1954; Osborne, 1955). Not all cases of epididymitis would have been attributable to *B. ovis* but it is probable that a significant proportion were. In addition, many rams that are infected with *B. ovis* do not develop lesions of epididymitis that can be detected by scrotal palpation (Hughes and Claxton, 1968; Murray, 1969).

In New Zealand, a survey of veterinarians conducted in 1981 suggested an estimated 4.2% of stud rams and 12.2% of commercial flock rams were infected with *B. ovis* (Liberona and Christiansen, 1981). In 1988, serological test data collated two years after the establishment of a voluntary *B. ovis* accreditation scheme showed that 4.6% of stud rams and 14% of commercial rams were seropositive when tested for the first time (Worthington and Hilbink, 1988). By 1996, 1.1% of stud rams and 5.6% of commercial flock rams tested were serologically positive (Reichel and West, 1997) although not all flocks in the country are tested and it is possible that the infection rate, particularly in commercial flocks, is higher than this figure suggests.

It is difficult to quantify the importance of *B. ovis* infection as the significance will vary depending on the infection rate and the management practices of each farm, and poor reproductive performance is often a multi-factorial problem. Potential financial losses that may occur due to ram infection with *B. ovis* stem from decreased ram fertility resulting in decreased ewe pregnancy rates. Ewes conceiving late in the breeding season due to poor ram fertility results in more lambs born late in the season, and these subsequently reach sale weight at a later date.

However, not all infected rams are affected to the same degree and some infected rams may have no demonstrable reduction in fertility compared with non-infected rams. McGowan and Devine (1960) and Swift and Weyerts (1970), took six and four infected rams, respectively, and one non-infected control ram, and mated each ram to a small number of ewes. Overall, ewes mated to infected rams had reduced lamb production although ewes mated to some of the infected rams had similar lambing results to the control groups. On three separate commercial farms, Middleberg (1973) mated infected and non-infected rams to separate groups of two-tooth ewes at a ratio of one ram per 50 ewes. On two of the properties there was no statistically significant difference between the pregnancy rates of ewes mated to infected or non-infected rams. However, on the third farm there were significant differences in the numbers of non-pregnant ewes and the number of lambs docked.

In a more recent study, Stobart et al. (1992) mated a group of 1050 ewes to 73 randomly selected rams, 26% of which were infected with *B. ovis*, to simulate an infected flock and a further 1050 ewes to 73 non-infected rams of good semen quality. This gives a ram to ewe ratio of one ram to 14 ewes. Ewes mated to the "infected" ram flock had a similar pregnancy rate compared with those mated to non-infected rams (91.1% and 95.2% respectively) but produced 16.9% fewer lambs at docking (80.1% and 97% respectively). The groups of ewes were managed separately during mating and lambing, but were grazed together during pregnancy. It was not stated whether the large discrepancy between pregnancy rate and docking percentage was due to late-term abortion, perinatal mortality or lamb death. No samples were collected from foetuses or dead lambs for bacteriology, and it can be concluded that there is no certainty that the difference in docking percentage between the two groups was attributable to *B. ovis* infection.

It is probable that in some flocks a *B. ovis* problem may be masked by "over-mating", where more rams than necessary are used to mate a flock of ewes. It has been demonstrated that rams are capable of mating at least 330 ewes within a 17 day period (Allison, 1975), yet most farmers in New Zealand use rams at a ratio of one per 50 to one per 100 ewes (Smith and Knight, 1998). "Over-mating" results in the same ewe being mated by multiple rams, so even if a proportion of rams have significantly

reduced semen quality due to *B. ovis* infection the ewe may become impregnated by another more fertile ram.

In flocks where abortion and neo-natal deaths occur, losses due to *B. ovis* infection are more tangible because aborted foetuses or dead lambs may be found. However, as outlined in Chapter 1.4.2, these manifestations of disease appear to be uncommon. It was concluded by Bruere (1973) that the main economic significance of infection was the wastage of stud ram hoggets and two-tooth rams, as infection renders them permanently unsound for sale. In addition, in infected commercial flocks there is the financial burden of replacing rams that develop epididymitis.

1.7 Control of *B. ovis* infection in sheep flocks

1.7.1 Eradication

Because transmission of *B. ovis* infection appears to require direct contact between infected and non-infected animals and there has been no evidence of transmission occurring by exposure to pasture previously stocked by infected animals (Buddle, 1955; Hartley et al., 1955; Keogh et al., 1958), removal of all infected rams and avoidance of re-introduction of infected rams should result in eradication of disease from a ram flock. Using this method, *B. ovis* infection was eliminated from Flinders and King Islands (Bass Straight, Australia) (Ryan, 1964), the Falkland Islands (Reichel et al., 1994) and individual flocks in New Zealand (West and Bruce, 1991). This suggests that maintenance of infection in ewes is unlikely to be a significant factor in the maintenance of infection in a flock.

Successful eradication programmes are based on scrotal palpation and serological testing of rams (Reichel et al., 1994). It is probable that a proportion of seropositive rams are not shedding *B. ovis* in the semen but instead are only transiently infected. However, in most situations, rams that are seropositive are culled.

Due to the incubation period of *B. ovis* infection and the fact that none of the available serological tests are 100% sensitive, serological testing on a single occasion is unlikely to detect all infected rams in a flock (Robles et al., 1998) and testing multiple times at intervals may be required. Difficulties in eradication of *B. ovis* from a flock can occur due to false-negative reactions in serological tests and in these cases a combination of serological tests and culture of semen may be necessary to identify all infected rams (West and Bruce, 1991; West et al., 1993).

Keogh et al. (1958) demonstrated that infected and non-infected rams can be maintained on a property without cross-infection provided they did not come into contact. Thus in situations where replacement of all infected rams is not financially feasible, a control option is to run a "two-flock system". Infected and non-infected rams are kept separate throughout the year, and the two groups of rams are mated to separate groups of ewes (Bruere and West, 1993).

1.7.2 Vaccination

Buddle (1954) investigated the efficacy of a number of experimentally prepared vaccines for use against *B. ovis* in sheep and found that simultaneous injection of killed *B. ovis* organisms suspended in saline-in-oil and live *B. abortus* Strain 19 organisms conferred good immunity. This finding was further validated both experimentally (Buddle, 1957b) and on farms (Buddle, 1958). Due to local tissue reactions from the saline-in-oil adjuvant, further work was undertaken investigating different adjuvants for suspension of the killed *B. ovis* organisms (Biberstein et al., 1962; Buddle, 1962). An aluminium hydroxide adjuvant killed *B. ovis* vaccine in combination with *B. abortus* Strain 19 was as effective as the protocol using a saline-in-oil vaccine and caused less severe local tissue reactions.

Problems with the vaccine protocol included shedding of *B. abortus* Strain 19 in the semen of some vaccinated rams (Buddle, 1962; Buddle et al., 1963), post-vaccination lameness with isolation of *B. abortus* Strain 19 from the lesions (Kater and Hartley, 1963; West et al., 1978), the development of *B. abortus* serological titres (Claxton, 1968) and the danger to human health in handling this vaccine.

Experiments were undertaken to develop a vaccine that did not involve the use of *B. abortus* Strain 19 and it was found that two doses of killed *B. ovis* suspended in saline-in-oil given at eight to 24 week intervals had a similar efficacy to the combined *B. ovis*/ Strain 19 vaccine (Buddle, 1962). This vaccine created significant subcutaneous lesions (Claxton, 1968; Quinlivan and Wallace, 1975) and it was suggested that intraperitoneal vaccination would prevent these lesions (Quinlivan and Wallace, 1975). However, research on different vaccination sites and protocols showed that intraperitoneal vaccination resulted in chronic granulomatous lesions in all tissues that the vaccine contacted (Bailey et al., 1988). Rams vaccinated by this route took longer to develop serological titres and appeared less resistant to experimental challenge compared with subcutaneous vaccination. It was also concluded that vaccination did not confer complete immunity and there was the possibility that natural challenge may result in infection of vaccinated rams (Bailey, 1986; Bailey et al., 1987).

In New Zealand, vaccination of rams against *B. ovis* was used in the 1970's and 1980's. However, use of the vaccine resulted in injection site lesions and because vaccination resulted in the development of serological titres (McDiarmid, 1978), it created difficulties in correctly identifying infected rams. A national accreditation scheme, based on testing and culling of rams, was launched in 1986 and in the late 1980's the vaccine was withdrawn from the market.

1.7.3 The New Zealand voluntary *B. ovis* accreditation scheme for sheep

The objective of a *B. ovis* accreditation scheme is to classify a ram flock as free of the disease. For a stud flock, this provides an assurance to ram purchasers that any rams they buy will not be infected whereas for a commercial flock, it is a way of ensuring that the flock remains non-infected. The concept of developing a New Zealand voluntary *B. ovis* accreditation scheme was initially presented in 1973 to the annual meeting of the Sheep Society of the New Zealand Veterinary Association (Bruere, 1973). At the same meeting Ris (1973), concluded that control based on detection using the CFT was technically feasible but uncertainties were expressed about whether such a scheme

would be economically justifiable. There was concern that the economic importance of *B. ovis* may not warrant an expensive scheme (Middleberg, 1973).

West (1978) outlined a proposal for a voluntary accreditation scheme and in 1979 West and Bruere (1979) presented the results of operating such a scheme in 14 stud and nine commercial flocks. The major sheep breed societies expressed interest in the scheme and it was concluded by the authors that nation-wide acceptance of such a scheme was an urgent priority for the sheep industry. Following this recommendation, Liberona and Christiansen (1981) undertook a cost-benefit study of an eradication scheme for *B. ovis* in New Zealand and concluded that a Government-funded eradication and accreditation scheme would not be economically justifiable.

In 1986 a national industry-based voluntary accreditation scheme, supported by the Ministry of Agriculture and Fisheries, was launched (Reichel and West, 1997). The scheme was based on annual scrotal palpation of all rams. Rams with lesions of epididymitis and a proportion of rams over the age of 15 months are tested serologically using the CFT. This scheme was primarily designed for stud flocks to ensure that commercial farmers could buy rams from accredited-free flocks, but some commercial farmers are also involved. All the costs associated with testing for each flock are borne by the owner. An outline of the scheme is included in Appendix 2.

In 1996, 1099 commercial flocks and 1394 stud flocks were registered on the scheme (Reichel and West, 1997). As at 30 June 1999 there were approximately 18,400 farms in New Zealand with 100 or more sheep (New Zealand Ministry of Agriculture and Forestry), suggesting that approximately 13.5% of commercial and stud farms are registered on the scheme. A higher proportion of stud farms compared with commercial farms are likely to be registered.

1.7.4 Therapy

Experimental antimicrobial treatment of *B. ovis* infected rams have been reported but there are no published field reports of treatment. Kuppuswamy (1954) artificially infected 24 rams with *B. ovis,* all of which were demonstrated to be excreting *B. ovis* in semen. Six rams were treated for 21 days with chlortetracycline and dihydrosteptomycin and all six stopped shedding for at least 113 days (16 weeks), with a significant improvement in sperm motility noted. Of six rams treated with sodium sulphamezathine and dihydrosteptomycin for 21 days, three discontinued shedding and of six treated with dihydrosteptomycin alone for 21 days, five discontinued shedding. The remaining six non-treated control rams continued to shed *B. ovis* in semen throughout the experiment.

Dargatz et al. (1990), attempted antibiotic treatment of 18 rams that were excreting *B. ovis* in semen with oxytetracycline and dihydrostreptomycin combinations for seven days to 15 days. Fifteen of the 18 rams stopped excreting *B. ovis* in semen for at least 21 weeks after treatment, although sera from all but two rams remained positive in a *B. ovis* ELISA. Treated rams had a significant improvement in sperm motility.

Unless a ram is valuable, treatment is unlikely to be economic (Kuppuswamy, 1954). Further, if treated, a ram would need to be isolated and re-tested both serologically and by culture of semen before use to ensure that the infection had resolved. For the purposes of the New Zealand voluntary accreditation scheme, any ram that is seropositive is deemed to be infected and therefore even if rams were treated and stopped shedding *B. ovis* in semen, the presence of serum antibodies would mean that the flock could not be accredited.

1.8 Background to the New Zealand deer industry

Eight species of deer, with the majority being Red deer (*Cervus elaphus),* were introduced to New Zealand between 1861 and 1910 and successfully established in the wild. Deer numbers increased rapidly and in the 1920's and 1930's deer became perceived as pests due to the damage caused to native forest and their contribution to erosion. Large-scale deer control operations began in 1931 and in 1956 they were officially classified as noxious pests (Challies, 1985).

Until about 1959 skins from feral deer were the main salable product but subsequently a venison industry was established with markets in Europe for wild shot deer. Deer farming was made legal in 1969 and the number of farmed deer has increased rapidly since then to an estimated population of two million in 2000 (New Zealand Game Industry Board). This is the largest farmed deer population of any country in the world. It is not uncommon for two-metre-high fencing to be erected on sheep and beef cattle farms to enable the farming of deer, and for sheep, beef cattle and deer to be farmed in conjunction with one another. Approximately 86% of the farmed deer population are Red deer (*Cervus elaphus*), with 10% Wapiti (*Cervus canadensis*) and Wapiti-cross and 4% Fallow deer (*Dama dama*) (Wilson et al., 1998). Feral deer are still present in moderate numbers throughout the country.

There are approximately 5000 deer farms in New Zealand with an average herd size of about 400 animals. The major products of the deer industry are venison and velvet antler. Farms may comprise of breeding properties for stud, weaner or venison production, finishing properties where weaners are finished to slaughter, or velvet properties where large groups of mixed-age stags are kept for velvet-antler production. Farms may run a combination of these enterprises.

In New Zealand the mating period or "rut" occurs in autumn from March to May. The gestation period of Red deer is approximately 233 days, with calving occurring in early summer from November to January (Wilson et al., 1998).

1.9 Isolation of *B. ovis* from deer

1.9.1 Introduction

In the early 1980s in Oklahoma in the United States of America, random sampling of stored sera from field-harvested white-tailed deer showed several to be positive in a *B. ovis* slide agglutination test (Barron, 1984). Unfortunately this finding does not appear to be well documented and numbers, age or gender of seropositive animals were not recorded. It was noted that in some geographical areas wild deer in Oklahoma share a common range with domestic sheep, suggesting a possible source of infection as *B. ovis* is present in sheep in the United States (McGowan and Shultz, 1956). Barron (1984) was subsequently able to demonstrate successful artificial infection of stags with *B. ovis.*

The first isolation of *B. ovis* from deer in New Zealand was at the end of the 1996 mating season when a Red deer stag that had been born in this country had semen collected for artificial breeding purposes. The semen appeared flocculent and was sent to a laboratory for analysis. It contained numerous leucocytes but no sperm, and bacteriological culture grew a pure growth of *B. ovis* (O'Neil, 1996; Bailey, 1997). Subsequently, infection has been documented in five commercial deer herds throughout New Zealand with lesions of epididymitis being detected in stags at slaughter (Scott, 1999).

1.9.2 Artificial infection in stags

Barron (1984) and Barron et al. (1985) reported artificial infection of white-tailed deer bucks with *B. ovis* by the conjunctival route using 1ml of an inoculum containing $5x10^9$ colony forming units/ml. Two mixed-age, and five six-month-old bucks were infected, resulting in serological titres that could be measured by a *B. ovis* CFT, and culture of the organism from the semen. Two bucks were slaughtered 77 days after inoculation and *B. ovis* was cultured from the epididymes of one buck. *Brucella ovis* was cultured from the epididymes of a buck infected 429 days previously, although there were no lesions in the reproductive tract.

West et al. (1999), artificially infected three nine-month-old Red x Wapiti stags by intravenous injection. Two stags maintained positive CFT titres and shed the organism in semen throughout the 10-month study period. The remaining stag did not shed the organism in semen, and serum from this stag was CFT negative or suspicious by day 110.

1.9.3 Natural infection in stags

Naturally-occurring infection has been reported on five commercial deer farms in New Zealand, with three located in Canterbury, one in Otago and one in Waikato (Figure 1.10.1). The first infected stag identified in New Zealand was discovered in Canterbury in June of 1996 following collection of semen for artificial breeding purposes (O'Neil, 1996). Despite an extensive trace-back investigation, the source of the infection was not determined (Scott, 1998). In May 1997, eight of 50 rising-three-year-old stags originally from the same property, but which had been grazing at another property since February 1997, were discovered at slaughter to have lesions of epididymitis. A further 34 stags from the same line were slaughtered five days later and sera from 30 of these were positive or suspicious in the CFT, indicating a very high rate of infection in this group of stags. Subsequently, the farmer decided to slaughter all 350 mixed-age velvetting stags on the property. Serological testing of these stags was not undertaken but six of the stags had palpable lesions of epididymitis, and *B. ovis* was isolated from one of these (Anon., 1997a; Anon., 1997b; Scott, 1998). The degree of contact between the groups of stags on the properties, and thus the possible source and spread of the disease, has not been reported.

In 1997 infection was confirmed in stags sent for slaughter from a property in Otago although details of this case are unknown (Scott, 1999). In July 1998, five of 20 rising-three-year old stags from another property in Canterbury, which had no known contact with the above properties, were found to be infected at slaughter but again the source of infection was not determined (Anon, 1998; Scott, 1999). In August 1998, infection was identified at slaughter in 30 of 60 two-year-old stags from the Waikato region. These stags had no known contact with deer from the Canterbury and Otago properties and

while the source of infection was not determined, it was suggested that it could have been from five rams grazing on the same farm (Scott, 1999).

Experimentally, Barron (1984) grazed an infected white-tailed deer buck with a non-infected buck for a 238-day period. At the end of this period, the non-infected buck was slaughtered and found to have lesions of epididymitis from which *B. ovis* was cultured, demonstrating transmission from stag to stag. West et al. (1999) grazed two infected rising-two-year-old stags with eight non-infected stags of the same age from September to June. Animals were blood sampled monthly and during April and May four of the eight control stags became infected. The timing of infection suggests that the mating period and sexual activity is important in the transmission of *B. ovis* between stags.

Thus, natural outbreaks have shown this disease to reach a high prevalence in groups of stags with sheep being the suspected source of infection. Artificial inoculation shows deer to be readily infected, providing a model for investigations in this thesis.

1.9.4 Natural infection in hinds

Barron (1984) mated an artificially infected buck that was shedding *B. ovis* in semen to six does. On day 76, sera from two does were positive in a *B. ovis* CFT but sera from all does were CFT negative on day 238. Only one fawn was born, and *B. ovis* was not isolated from the liver, lung, spleen or stomach contents of this fawn. It is unknown how many of the six does were actually mated by the infected stag but the authors considered that the poor pregnancy rate may have been from poor fertility of the buck due to *B. ovis* infection.

The initial infected stag in New Zealand had been mated to 42 hinds during March, April and May of 1996. In July 1996, serum from one hind was positive in the CFT (titre 4/16) and suspicious in the ELISA and GD. At further sampling three weeks later serum from this hind was suspicious in the CFT (titre 1/8), high negative in the ELISA and negative in the GD. All the other hinds were seronegative (Scott, 1999). It was not reported whether any of the hinds aborted or had dead calves.

1.9.5 Transmission of infection from stags to rams

West et al. (1999), grazed 10 non-infected rams with two infected stags for a 10-month period. Rams were blood sampled for testing in the *B. ovis* CFT, and semen was collected on three occasions for bacteriological culture. None of the rams became infected with *B. ovis* suggesting that transmission from stags to rams may be a low-risk event.

1.10 Prevalence and geographical distribution of *B. ovis* infection in deer in New Zealand

One thousand and seventy four deer serum samples, representing less than 0.1% of the New Zealand farmed deer population, have been submitted to diagnostic laboratories specifically for testing for *B. ovis* infection using the CFT (Table 1.10.1). These sera were from properties where the submitting veterinarian suspected *B. ovis* infection in the deer, or from deer that were routinely tested as part of export regulations. Using the cut-off values described for sheep sera, 118 serum samples or 11% of the sera tested were positive or suspicious in the CFT. Seven hundred and sixty eight sera were submitted from five properties on which infection was confirmed by culture of semen or epididymes (Chapter 1.9.3; Figure 1.10.1) and 106 sera from these properties had positive or suspicious CFT titres. The remaining 12 positive or suspicious samples were from six properties where no attempt has been made to confirm the presence of infection by bacteriological culture. Therefore it is unknown whether they represent actual infections or false-positive reactions.

Of 1498 deer serum samples submitted to diagnostic laboratories for other reasons and tested in the *B. ovis* CFT, six were positive but at least four of these were considered to be false-positive reactors (Kittelberger and Reichel, 1998).

Since 1998, only one new case of *B ovis* infection in deer has been detected by serological testing and no new cases have been confirmed by bacteriological culture. Scrotal palpation of stags to detect lesions of epididymitis, with subsequent investigation of any lesions identified, is a routine postmortem inspection procedure at deer slaughter premises. It would appear that while the exact prevalence of *B. ovis* infection in the New Zealand farmed deer population is not known, it is likely to be low.

Table 1.10.1 A comparison of total deer numbers as at 30 June 2000, number of serum samples from deer tested for *B. ovis* infection, number of seropositive samples, numbers of properties tested and number of properties on which seropositive serum samples were collected, by regions of New Zealand.

Region	Estimated number of farmed deer as at 30 June 2000	Number of serum samples tested from deer	Number of test-positive serum samples	Number of properties from which serum samples were tested	Number of properties tested with test-positive serum samples
Northland	36,000	0	0	0	0
Auckland	34,000	0	0	0	0
Waikato	190,000	21	14	3	1
Bay of Plenty	89,000	14	0	2	0
Gisborne	31,000	0	0	0	0
Hawkes Bay	135,000	97	0	1	0
Taranaki	17,000	0	0	0	0
Manawatu-Wanganui	184,000	8	1	2	1
Wellington	34,000	3	0	1	0
Tasman	43,000	4	0	2	0
Marlborough	26,000	3	0	1	0
West Coast	42,000	0	0	0	0
Canterbury	535,000	787	80	7	3
Otago	186,000	95	18	4	3
Southland	472,000	39	4	4	2
TOTAL	**2,053,000**	**1074 (0.05% of total farmed deer) population)**	**117 (11% of deer tested)**	**27 (0.5% of total deer farming properties)**	**10 (42% of properties tested)**

Source: Statistics New Zealand; New Zealand Game Industry Board, AgriQuality Serology

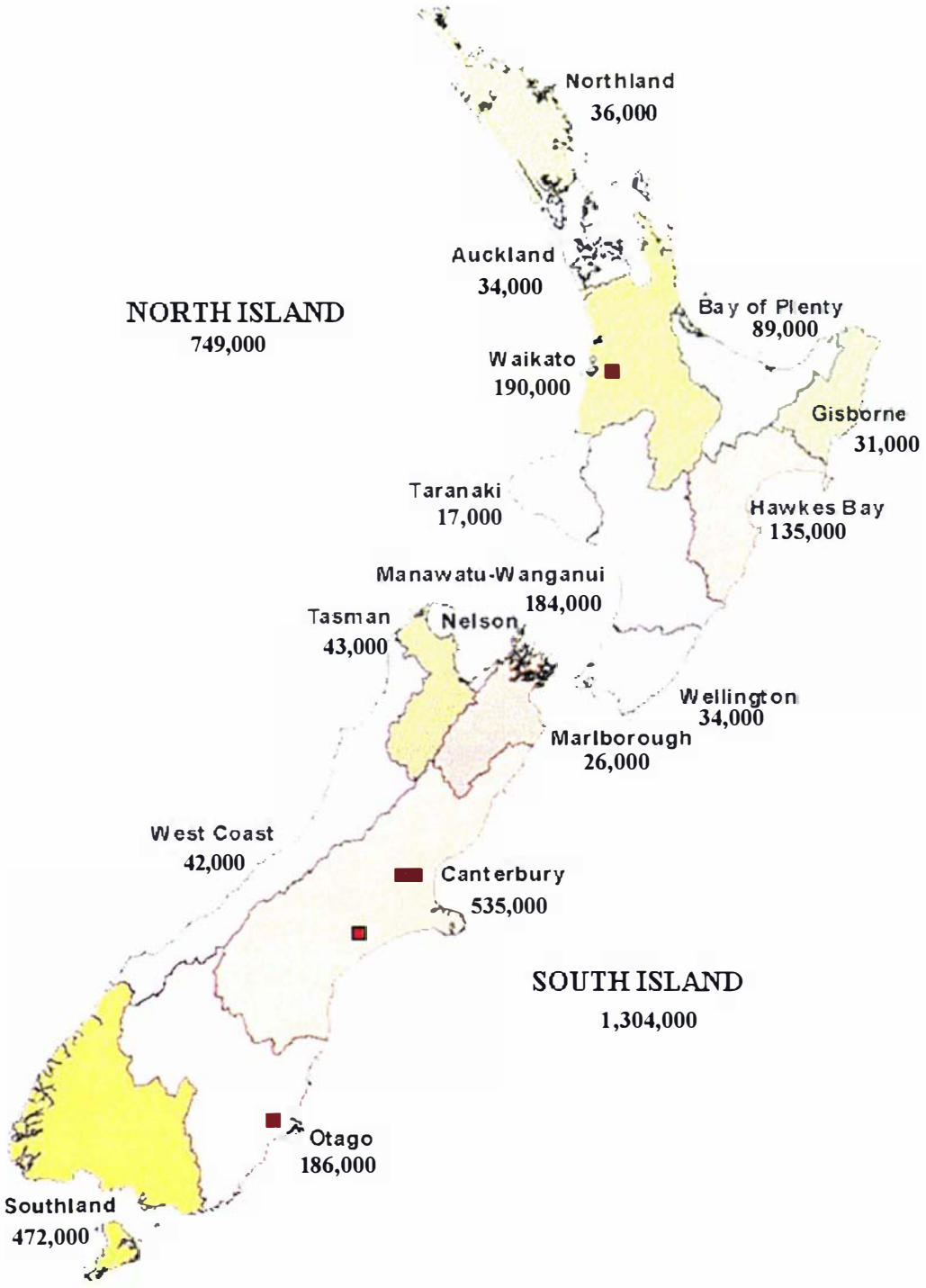


Figure 1.10.1 Map of New Zealand showing geographical regions and number of deer per region. Red squares indicate the approximate location of farms on which infection has been confirmed in stags by culture of *B. ovis* from semen or epididymes.

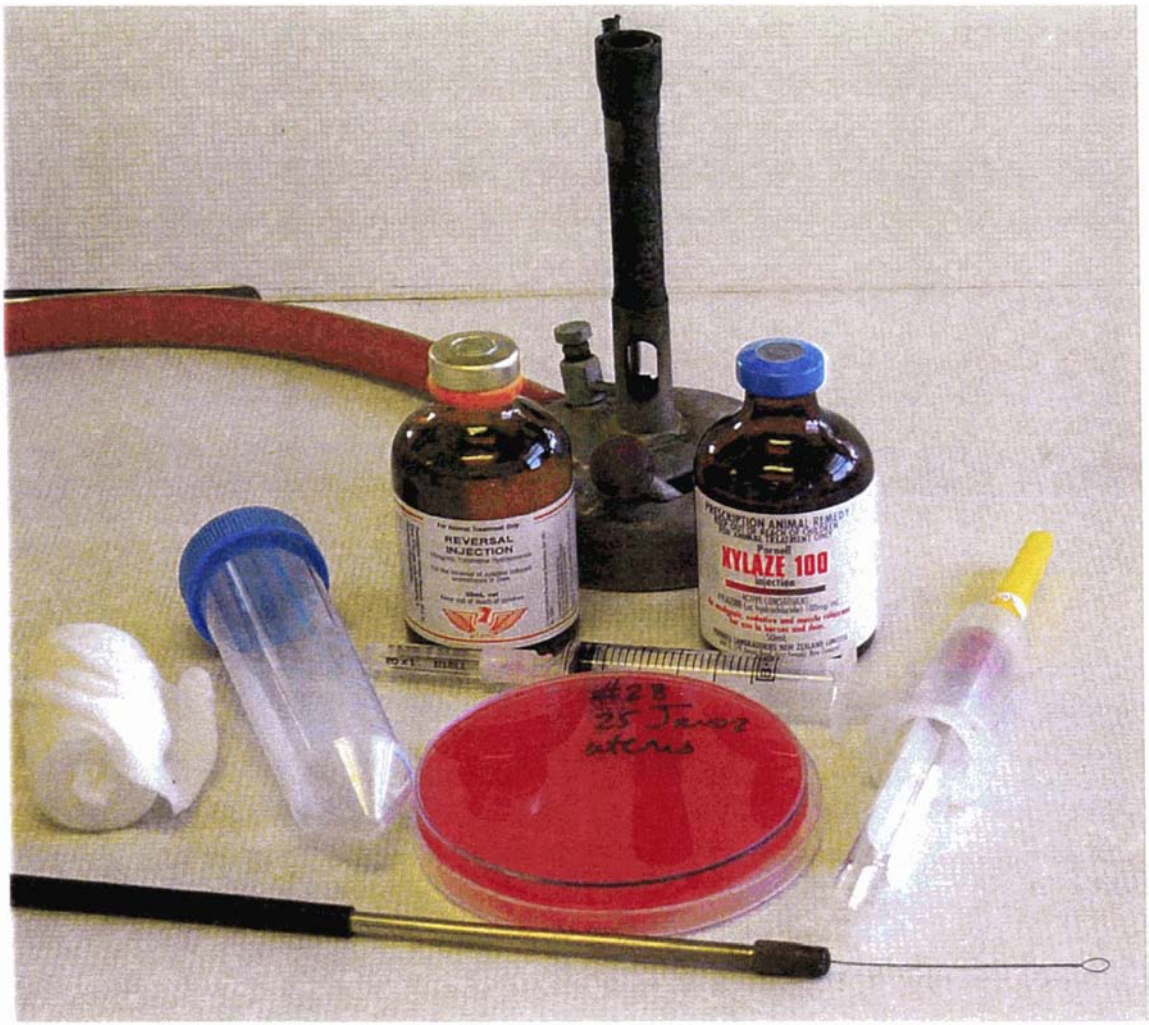
1.11 Objectives of the present study

At the beginning of this Ph.D. project in January 1999 there was no available data on the prevalence of *B. ovis* infection in New Zealand farmed deer. Little data was available on the epidemiology of the disease but it appeared that in some groups of stags the group-prevalence could be as high as 80 to 90% (Scott, 1999). Little was known about the transmission and effects of the disease in deer and while tentative control guidelines were proposed to the deer industry (West et al., 1998), these were based on knowledge of the disease in sheep. Because of the high prevalence in some naturally infected herds, it was considered that this apparently emerging disease could have significant consequences to the deer industry if it became widespread. At that time, it was unknown whether more cases would arise. A need was identified, both by the New Zealand deer industry and veterinarians, to further research the disease in deer (West et al., 1999b). Therefore this Ph.D. project was undertaken with the objectives of investigating:

1) strain differences in *B. ovis* isolated from commercial rams and commercial stags
2) transmission from rams to stags grazing in the same paddock
3) transmission between stags by indirect contact (alternating grazing or grazing in adjacent paddocks)
4) possible mechanisms of transmission between stags
5) establishment and persistence of infection in stags
6) pathological, histological and microbiological changes in the reproductive tract of stags
7) effectiveness of serology as a diagnostic test for use in deer
8) effects of infection on stag semen quality
9) effects of infection on hind reproductive performance
10) venereal transmission

Chapter Two

General materials and methods



2.1 Introduction

Materials and methods that are common to all experiments are included in this chapter. Materials and methods that are specific to certain experiments are described in the chapter or section detailing that experiment.

2.2 Animals

2.2.1 Experimental site

With the exception of the behavioural observations detailed in Section 4.5, all experiments using deer were conducted at the Massey University Deer Research Unit located on Sheep Farm Road, Palmerston North. This is a 40 hectare flat to rolling breeding and finishing property, all of which is deer-fenced. Apart from occasional yearling cattle that are bought on to control the pasture, deer are the only species farmed on this property. A stud stag is purchased every 2 to 3 years, otherwise no deer are introduced onto the property. The neighbouring properties farm sheep, goats or cattle but no deer.

For an experiment involving rams (Chapter 5), the rams were grazed on the Massey University Large Animal Teaching Unit hospital block, which is one hectare in size. The only other animals grazed on this land are horses, and it is surrounded by the Massey University campus.

2.2.2 Experimental animals

All deer used in these trials were Red or Red-cross-Wapiti deer bred on the Deer Research Unit. Management practices were standard for a commercial deer farm and all deer were grazed on pasture throughout each year, with some hay supplementation during the winter months.

All rams used in these trials were Romney or Romney-cross sourced from commercial farms. Management practices were standard for a commercial sheep farm.

2.2.3 Timing of Experiments

Experiments were undertaken between January 1999 and November 2001. Within each chapter, the start day of the actual experiment is defined as Day 0. Where preparation before the actual experiment was required, this time has been defined as negative days.

2.2.4 Animal ethics

All experimental procedures involving the use of animals that were undertaken during the course of this study had animal ethics approval from the Massey University Animal Ethics Committee, Palmerston North, New Zealand.

2.3 Sample collection

2.3.1 Collection of semen from stags by electro-ejaculation

Stags were sedated with 1mg/kg bodyweight Xylazine (Parnell Xylaze 100, Parnell Laboratories New Zealand Ltd.) by intramuscular injection. Once recumbent, stags were dragged onto rubber mats with their heads and down-side limbs supported by pillows of hay and restrained in left lateral recumbency with the uppermost hind limb extended cranially and restrained with a soft rope. The penis was extruded and held with a gauze bandage, and electro-ejaculation achieved using a lubricated rectal probe 25mm in diameter and 200mm long with three electrodes placed 10mm apart on the ventral side. The electro-ejaculator was powered by a Pulsator III Z battery (Lane Manufacturing Inc., Denver, Colorado, USA). Semen was collected into a 50ml sterile collection pottle (Greiner Labortechnik, Frickenhausen, Germany). Sedation was reversed using 0.25mg/kg bodyweight Yohimbine (Reversal injection, Phoenix Pharm Distributors Ltd.) injected into the jugular vein. Semen was plated onto culture medium within four hours of collection using a sterile cotton-tipped swab.

2.3.2 Collection of semen from rams by electro-ejaculation

Rams were not sedated and were restrained in left lateral recumbency on a low table with their hindlimbs extended slightly caudally. The penis was extruded and held with a gauze bandage and electro-ejaculation achieved using a lubricated self-contained electro-ejaculator inserted into the rectum (Ram probe Mk V, AJ Thompson Manawatu Ltd., Palmerston North). Semen was collected into a sterile 50ml collection pottle (Greiner Labortechnik, Frickenhausen, Germany). Semen was plated onto culture medium within four hours of collection using a sterile cotton-tipped swab.

2.3.3 Collection of post-mortem samples at slaughter

Deer were slaughtered at Venison Packers, Feilding, New Zealand, and rams were slaughtered at Country Meats, Palmerston North, New Zealand, using a captive bolt to the head followed by exsanguination. Following pelt removal, the animals were eviscerated before the carcass was processed. Blood samples were collected by free-catch into Vacutainer® tubes at the time of exsanguination. Tissues from the reproductive tract were collected into labeled plastic bags following evisceration and dissected in the Massey University post-mortem room. Specimens for *B. ovis* culture were flamed using ethanol, incised, and the inside of the tissue swabbed with a sterile cotton-tipped swab which was plated onto culture medium.

2.3.4 Collection of samples from the prepuce of rams and vagina of hinds

Samples were collected by moistening a sterile cotton-tipped transport swab (BBL cultureswab plus™, Amies without charcoal, Becton Dickinson, New Jersey, USA) in sterile saline and rubbing it across the area to be sampled, taking care to avoid excessive contamination. The swabs were immediately put in transport media, and plated onto culture media within four hours of collection.

2.4 Microbiology

2.4.1 Microbiological methods

Preparation of inoculum

All the *B. ovis* suspensions used for artificial inoculation of deer and rams came from an isolate of *B. ovis* that was isolated from a stag at Lincoln, in Canterbury, in 1996. This isolate was frozen and stored at -70°C. When required, a small volume of isolate was defrosted, plated onto a blood agar plate and incubated at 35°C in an environment containing 8% CO_2 for three days. Provided a pure growth of an organism morphologically resembling *B. ovis* grew, one colony from this was used to plate each of 8 blood agar plates which were incubated as above for a further three days. Depending on how heavy the growth on each plate was and the volume of inoculum required, the growth from two to eight plates was transferred to the appropriate volume of sterile saline to make an inoculum the transparency of McFarlane Standard five and a spectrophotometer (Helios α Basic, BioLab, Auckland, New Zealand) reading at wavelength 550nm between 1.00 and 1.25.

The inoculum was diluted 10-fold nine times by mixing 0.1 ml inoculum with 0.9 ml of sterile saline, then transferring 0.1 ml of this to a further 0.9 ml sterile saline and so on. 0.1 ml of sixth, seventh, eighth and ninth 10-fold dilutions were plated in triplicate onto blood agar plates and incubated for three days. The number of colonies on plates for each dilution were counted and averaged to determine the number of colony-forming units per ml of inoculum.

Bacteriological culture procedures

Within 24 hours of collection, samples were plated onto *B. ovis* selective media and blood agar using a sterile cotton-tipped swab. Plates were incubated at 35°C for five days in an environment containing 8% CO_2. For bacterial colonies resembling *B. ovis*, one colony was transferred to a blood agar plate and incubated as above for a further three days. Provided the growth on this plate was pure, growth was transferred into a Heart Infusion broth which was used to inoculate Simmon's Citrate and urea media, blood and McConkey agar plates and nitrate formula, and these were incubated as above

for a further three days. The blood agar was examined to ensure that growth of the organism was pure, and identification of *B. ovis* was made on the basis of its ability to grow on selective media, colony morphology (slow-growing, circular, glistening grey colonies; Figure 2.1), presence of Gram negative cocco-bacilli on Gram stain, presence of red acid-fast organisms on modified Ziehl-Neelson stain, no reaction in the Simmon's citrate, urea, nitrate or oxidase tests and failure to grow on McConkey agar.

2.4.2 Media, reagents and stains

Addresses of media manufacturers
BBL™, Becton Dickinson, Franklin Lakes, NJ, USA,
Difco, Detroit, MI, USA.
New England Biolab (NEB), Beverley, MA, USA.

Selective media for isolation of B. ovis (modified Thayer-Martin media)
Dissolve 36g of GC medium base (BBL) in 600ml of distilled water, and dissolve 10g of Haemoglobin (BBL) in 400ml of distilled water. Autoclave both mixtures and allow to cool to 57°C. Dissolve the contents of a 10ml vial of VCN inhibitor in 10ml of sterile distilled water. Either dissolve 10mg of sodium nitrofurantoin in 1ml of sterile distilled water, or 10mg of nitrofurantoin in 1ml of acetone. Combine all the ingredients and mix thoroughly, keeping the temperature at 57°C. Pour into sterile petri dishes.

Blood agar
To make the salt base for the bottom layer, mix 15g agar, 5g NaCl and 1 litre of distilled water. Pour 10ml into each sterile petri dish. To make the blood base for the upper layer, mix Columbia blood agar base (Difco) and autoclave. Cool to 45°C and add 5% defibrinated sheep's blood. Mix and pour 10-15ml over the salt base in each petri dish.

McConkey agar and Tryptic Soy Agar (TSA)
For each, make media according to the manufacturers directions (Difco). Autoclave for 15 min at 121°C and pour into sterile petri dishes.

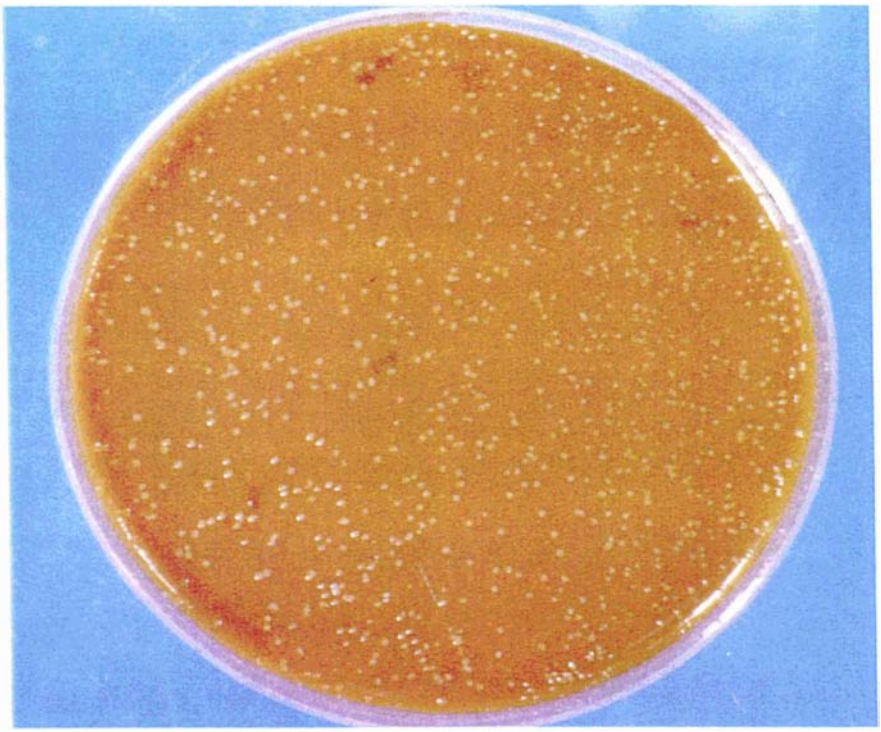


Figure 2.1 Circular, glistening grey *B. ovis* colonies growing on selective media.

Heart Infusion Broth (HIB)

To make, mix 25g Heart Infusion broth powder (Difco) in 1 litre of distilled water and autoclave for 15 min at 121°C.

Citrate utilisation test

To make, mix 24.2g Simmons citrate agar (Difco) in 1litre distilled water, pour 3ml into each bijoux jar and autoclave for 15 min at 121°C. Allow to slope during cooling. To inoculate the media, use a straight wire to streak the organism-in-HIB suspension onto the slope of the media. Incubate at 37°C in 8% CO_2 for 3 days. A positive reaction is indicated by growth of the organism on the slope, and the development of an intense blue colour on the slope. *Brucella ovis* gives no reaction.

Nitrate reduction test

To make the test reagent, mix 25g Heart Infusion broth powder (Difco), 2g potassium nitrate CP and 1 litre distilled water. Adjust the pH to 7.0 and aliquot 4ml into each bijoux bottle with Durham tubes. Autoclave for 15 min at 121°C. To inoculate, add 4ml of organism-in-HIB suspension and incubate at 37°C in 8% CO_2 for 3 days. Look for gas in the Durham tube – if present and the organism is a non-fermenter of glucose then the test is positive. If no gas is present, add 0.2ml Nitrate A reagent (containing 0.2g 5-amino 2-naphthalensesulfonic acid, 30ml glacial acetic acid and 120ml distilled water) and 0.2 ml Nitrate B reagent (containing 0.5g sulfanilic acid, 30ml glacial acetic acid and 120ml distilled water). Development of a red colour within 1-2 minutes indicates that nitrate has been reduced to nitrite. If no colour develops, add 20mg zinc oxide powder. If a red colour develops it indicates that nitrate is present and the test is negative. If no colour develops, it indicates that the organism has reduced nitrate to nitrite, and then nitrite to non-gaseous products. *Brucella ovis* does not produce gas or reduce nitrate.

Oxidase

Place a drop of oxidase reagent (Becton Dickinson) onto a piece of filter paper and then use the moistened filter paper to swab the organism to be tested. Within 1 minute, a positive test turns a purple-black colour. *Brucella ovis* gives no reaction.

Urea slopes

To make the agar, mix 15g bacto agar in 1l distilled water and autoclave for 15 min at 121°C. Make urea base by mixing 29g urea agar base (BBL) with 100ml distilled water and sterilise by filtration. When the melted agar is cooled to around 37°C, mix 200ml with 20ml of urea base. Pour 3ml into each bijoux bottle and allow to slope during cooling. To inoculate the media, add 4 drops of the organism-in-HIB suspension onto the slope of the media using a sterile pipette. Incubate at 37°C in 8% CO_2 for 3 days. A positive reaction turns the colour of the media from yellow to pink. *Brucella ovis* gives no reaction.

Gram stain

Fix the slide using gentle heat. Flood with 0.5% Methyl violet for 30 seconds, rinse, flood with Lugol's iodine for 30 seconds and then rinse again. Flood the slide with actone and then rinse immediately. Flood the slide with 0.5% Safranin for 1 minute, rinse and air-dry. *Brucella ovis* is Gram negative and should stain pink, and can be visualized at 1000x magnification with a light microscope.

Modified Ziehl-Neelson stain

Flood slide with dilute Carbol Fuchsin for 10 minutes. Rinse with water and then decolourise with 0.5% Acetic acid for 15 seconds. Rinse and counterstain with 1% Methylene Blue for 30 seconds. Rinse and air-dry. *Brucella ovis* is acid fast and should be red, and can be visualized at 1000x magnification with a light microscope. Other organisms will stain blue.

Pulsed-field gel electrophoresis buffers

0.5M EDTA, pH 8.0

Dissolve 18.6g di-sodium EDTA in 70ml MilliQ water and adjust the pH to 8.0 with NaOH pellets. Let the buffer equilibrate for at least 5 minutes and recheck the pH. Make up the volume to 100ml with MilliQ water and autoclave.

1M Tris-HCl. pH 8.0

Dissolve 12.1g of Tris base in 80ml MilliQ water and adjust the pH to 8.0 using concentrated HCl. Let the buffer equilibrate for at least 5 minutes and recheck the pH. Make up the volume to 100ml with MilliQ water and autoclave.

PETT IV buffer

To make 100ml of buffer, mix 20ml 5M NaCl, 1ml 1M Tris-HCl and 2ml 0.5M EDTA. Make up to 100ml with MilliQ water and autoclave.

TE buffer. 10:1

To make 100ml of buffer, mix 1ml 1M Tris-HCl and 200µl 0.5M EDTA and make up to 100ml with MilliQ water. Autoclave.

TBE buffer. 5x

To make 2 litres of buffer, mix 108g of Tris, 55g Boric aci, 40ml 0.5M EDTA and make up to 2l with MilliQ water. Autoclave.

Lysis buffer

To make 100ml of buffer, mix 10ml 0.5M EDTA, 5ml 1M Tris-HCl, 1g Sodium lauroyl sarcosine (1%), 100mg of Proteinase K (0.1%) and 95ml of MilliQ water. Buffer can be stored at -20°C until use.

Restriction buffer (*Swa*I)

Add 12µl of restriction buffer (NE buffer 3, NEB), 10µl of BSA and 78µl sterile MilliQ water to each plug and mix.

Cutting buffer

Mix 10µl restriction buffer (NE buffer 3, NEB), 10µl of BSA, 30 units of *Swa*I (NEB) and make up to 100µl with MilliQ water. Add to each plug and mix.

2.5 Serological Testing

2.5.1 Blood sampling

Blood samples were collected from the jugular vein into 10ml blood tubes containing no anti-coagulant (Vacutainer® No Additive, Becton Dickinson, Franklin Lakes, NJ, USA). Between one and four hours after collection, samples were centrifuged at 3200 rpm for 10 minutes and serum was transferred into 1ml micro-tubes. Within one day of collection, serum samples were couriered to AgriQuality Serology, Palmerston North, New Zealand.

2.5.2 Complement Fixation Test (CFT)

A cold *B. ovis* CFT was performed in microtitre plates using the method described by Anon (1983). Doubling serum dilutions of either 1:4 to 1:128, or 1:4 to 1:2048 were tested and the results recorded as the strength of the reaction (1-4) at the highest serum dilution with that reaction. During this study, titres of less than 4/4 were considered negative, titres from 1/8 to 3/8 were considered suspicious and titres equal to or greater than 4/8 were considered positive.

When CFT titres were represented on a graph they were converted to a CFT "score" for ease of presentation. Each titre was sequentially assigned a number so that no reaction in the CFT equated to a score of 0, a 1/4 reaction gave a score of 1, a 2/4 reaction gave a score of 2 and so on. Thus the cut-off value for suspicious reactions of 1/8 equates to a score of 5 and the cut-off value for positive reactions of 4/8 equates to a score of 8.

2.5.3 Enzyme-linked Immunosorbent Assay(ELISA)

A *B. ovis* ELISA test was performed using the method described by Worthington et al. (1984). Absorbance values were converted into ELISA units using the method described by Reichel et al. (1999). During this study, titres less than 75 were considered negative, titres between 76 and 150 were considered suspicious and titres greater than 150 were considered positive.

2.6 Histopathology

Sections of tissue were fixed in a 10% formalin solution, trimmed and processed routinely in a tissue processor (Leica Jung, 1050). Tissues were then embedded in paraffin (Paraplast, Oxford Labware, St Louis, MO, USA) and cut to a thickness of three microns on a microtome (Microtech rotary, 4055). Sections were stained with Haemotoxylin and Eosin using the method described by Sheehan and Hrapchak (1980), mounted in Entellan mountant media (Merck, Darmstadt, Germany) and examined using a light-microscope at 400x magnification.

Chapter Three

Strain typing *Brucella ovis* isolates by pulsed-field gel electrophoresis

3.1 Introduction

Historically, different strain types of *B. ovis* have not been recognised by bacteriologists. O'Hara et al. (1985) isolated 33 field strains of *B. ovis* from six different farms and subjected them to a number of restriction endonucleases. They found three restriction endonucleases that showed clear DNA fragment patterns, but they did not find any differences in *B. ovis* strains. Bailey and West (1987) compared four ram field isolates, the Type strain and two vaccine strains of *B. ovis* using the restriction endonuclease *Eco*R_I. No genetic differences were detected. A personal communication referenced in that paper noted that Australian workers had not detected genetic differences in 12 *B. ovis* isolates using an isoenzyme electrophoresis technique. Halling and Zehr (1990) undertook Southern blotting of highly repeated DNA from 12 *B. ovis* isolates from New Zealand and America isolated over a 30-year period and found similar hybridisation patterns between isolates. Random amplification of polymorphic DNA (RAPD) analysis of five *B. ovis* isolates suggested that there were differences between isolates, but the degree of similarity between the isolates was not identified (Tcherneva et al., 2000). It has been shown that the RAPD method of analysis can give inconsistent results (Meunier and Grimont, 1993; Tyler et al., 1997).

Strain typing of bacterial organisms can be undertaken by a variety of techniques including restriction endonuclease (RE) and pulsed-field gel electrophoresis (PFGE). With these techniques the bacterial DNA is cut into fragments using an endonuclease, and then an electrical field separates the fragments of DNA which can be visualised as "bands" according to their different molecular weights. PFGE differs from RE in that the orientation and duration of the electric field changes periodically rather than being fixed (Birren and Lai, 1993). An advantage of PFGE is that it can separate DNA fragments up to several megabases in length, increasing the analytical power (Lahti, 1996).

McGillivery et al. (1988) suggested that RE analysis of *Brucella* species using high-frequency cleavage enzymes was of limited use in differentiating strains and Jensen et al. (1999) suggested that PFGE using low-frequency cleavage enzymes gave better

discrimination between species. A number of researchers have undertaken PFGE analysis of *Brucella* species and have demonstrated that this technique is useful for differentiating between species of *Brucella* (Allardet-Servent et al., 1988; Jensen et al., 1995; Jensen et al., 1999). These authors all performed PFGE on *B. ovis* but only one isolate was used by each, thus differentiation between *B. ovis* isolates was not possible.

In 2000, PFGE of *B. ovis* isolates using the enzyme *Swa*I revealed three different banding patterns, suggesting different strains (MJ Leyland, *pers. comm.*). The objective of the experiment reported in this chapter was to perform PFGE on field isolates of *B. ovis* from rams and stags to determine whether rams and stags are infected by the same strain of *B. ovis*.

3.2 Materials and methods

General sample collection and analysis methods are described in Chapter 2.

3.2.1 Isolates of *B. ovis*

Ram isolates

A total of 10 *B. ovis* field isolates from commercial flock rams from 10 different farms in New Zealand were analysed. These isolates were collected from the Bay of Plenty, Hawkes Bay, Manawatu, Waikato and Wellington regions of the North Island and the Marlborough region of the South Island (Table 3.2.1, Figure 3.2.1). Isolates were collected from live rams by culture of semen or from slaughtered rams by culture of epididymes, and stored at -70°C until use.

Stag isolates

Two *B. ovis* isolates were collected from infected stags. One isolate was collected in 1996 from a stag in the Canterbury region of the South Island by culture of semen. The other isolate was collected in 1997 from a stag in the Waikato region of the North Island by culture of epididymes at slaughter (Table 3.2.1, Figure 3.2.1). These isolates were stored at -70°C.

Type strain

The Type strain of *B. ovis,* NCTC 10037, was obtained from Environmental and Science Research (ESR, Wellington, New Zealand). The Type strain had been isolated from a ram in New Zealand in 1956 (Buddle, 1956), but the regional origin of this strain does not appear to be known.

Table 3.1 Electrophoresis lane, animal species, region (and island) and year of isolation for isolates of *B. ovis* analysed by PFGE.

Lane	Species	Region (and island)	Year
1	Ovine	Waikato (NI)	1991
2	LOW MOLECULAR WEIGHT MARKER		
3	Ovine	Wellington A (NI)	2000
4	Ovine	Wellington B (NI)	2001
5	Ovine	Manawatu A (NI)	2000
6	Ovine	Marlborough (SI)	2000
7	Ovine	Manawatu B (NI)	2001
8	Ovine	Bay of Plenty (NI)	2000
9	Ovine	Hawke's Bay (NI)	2000
10	Ovine	Manawatu C (NI)	1998
11	Ovine	Type strain	1956
12	Ovine	Wellington C (NI)	1990
13	LAMBDA MARKER		
14	Cervine	Waikato (NI)	1997
15	Cervine	Canterbury (SI)	1996

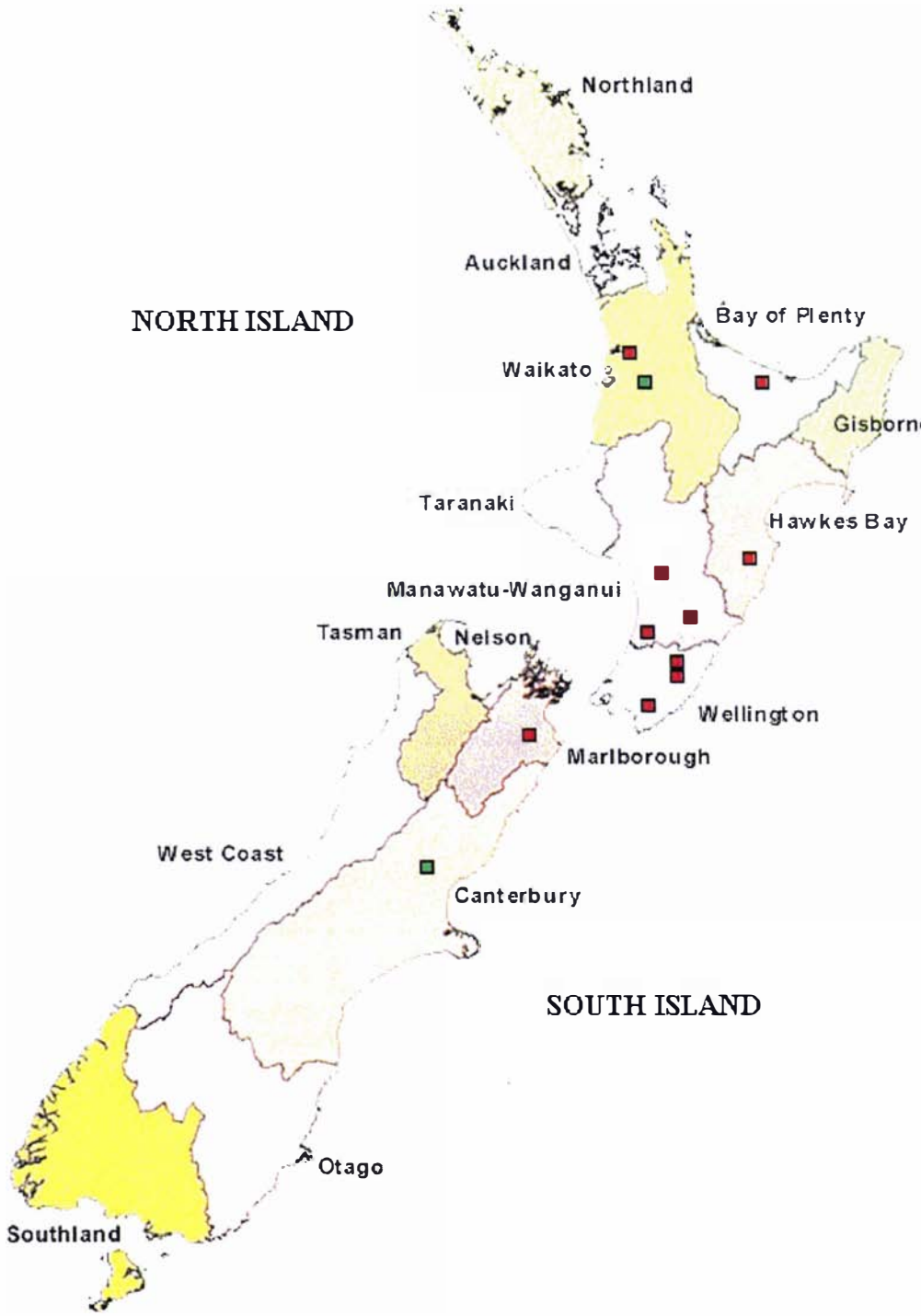


Figure 3.1 Regional map of New Zealand showing the approximate location of farms from which field isolates of *B. ovis* were obtained. Red squares denote ovine isolates and green squares denote cervine isolates.

3.2.2 Pulsed field gel electrophoresis of *B. ovis*

Pulsed field gel electrophoresis was undertaken using a method developed by MJ Leyland (*pers. comm.*), which was adapted from a method described by Böhm and Karch (1992). Details of the media and buffers used are described in Section 2.4.

Plug preparation

Growth off a 65 hour TSA plate was harvested into brain-heart infusion broth and the volume of cells equivalent to 150µl of cells with an optical density of 1.4 at 610nm was centrifuged at 13000 rpm for five minutes. The supernatant was removed and cells were re-suspended in cold PETT IV buffer. This step was repeated. Molten agarose gel was added to the cell suspension, mixed, dispensed into plug moulds and cooled to allow the plugs to set. Plugs were then placed in lysis buffer and incubated at 56°C for 15 hours. Plugs were washed with TE buffer five times at hourly intervals to remove all lysis buffer.

Restriction digest

A third of each plug was sliced off and equilibrated in restriction buffer (*Swa*I) for 45 minutes before being replaced with cutting buffer. Plugs were incubated at 25°C for 12-24 hours. The remaining two-thirds of each plug was stored for future use.

Pulsed field gel electrophoresis

A gel mould was prepared using 1% PF Agarose and allowed to solidify. The gel tank was filled with 0.5x TBE buffer and the gel was pre-electrophoresed for one and a half hours. Plugs and markers were loaded into the gel and this was run at 6V/cm for 22 hours with an initial switch time of five seconds and a final switch time of 30 seconds. At the conclusion of this period the gel was stained in ethidium bromide for 10 minutes, rinsed in MilliQ water and an image was captured under UV illumination by the Gel Doc 2000 system (Bio-Rad Laboratories, Hercules, CA, USA). The PFGE patterns were analysed by Diversity Database software (Bio-Rad Laboratories, Hercules, CA, USA) and similarities between the PFGE patterns were calculated by the Dice correlation coefficient with a maximum position tolerance of 1%. A dendrogram was constructed by the unweighted pair group method using arithmetic averages.

3.3 Results

Ten of the 13 *B. ovis* isolates, including eight ovine isolates from the North Island, one cervine isolate from the North Island and the Type strain, had identical banding patterns (lanes 1, 3-5, 7-11 and 14, Figure 3.2). This represents a strain of *B. ovis* that has been named Type A. An ovine and a cervine field isolate, both from the South Island, showed a banding pattern that was different at five bands to Type A (Figure 3.2, lanes 6 and 15; Figure 3.3) and dendrogram analysis showed that the two patterns were only 78% similar (Figure 3.4). This represents a second strain of *B. ovis*, which has been named Type B. An ovine isolate from Wellington collected in 1990 had one less band than Type A (Figure 3.2, lane 12; Figure 3.3) and by dendrogram analysis was shown to be 97% similar to Type A (Figure 3.4). It is considered to be a sub-type of Type A and has been named Type A1.

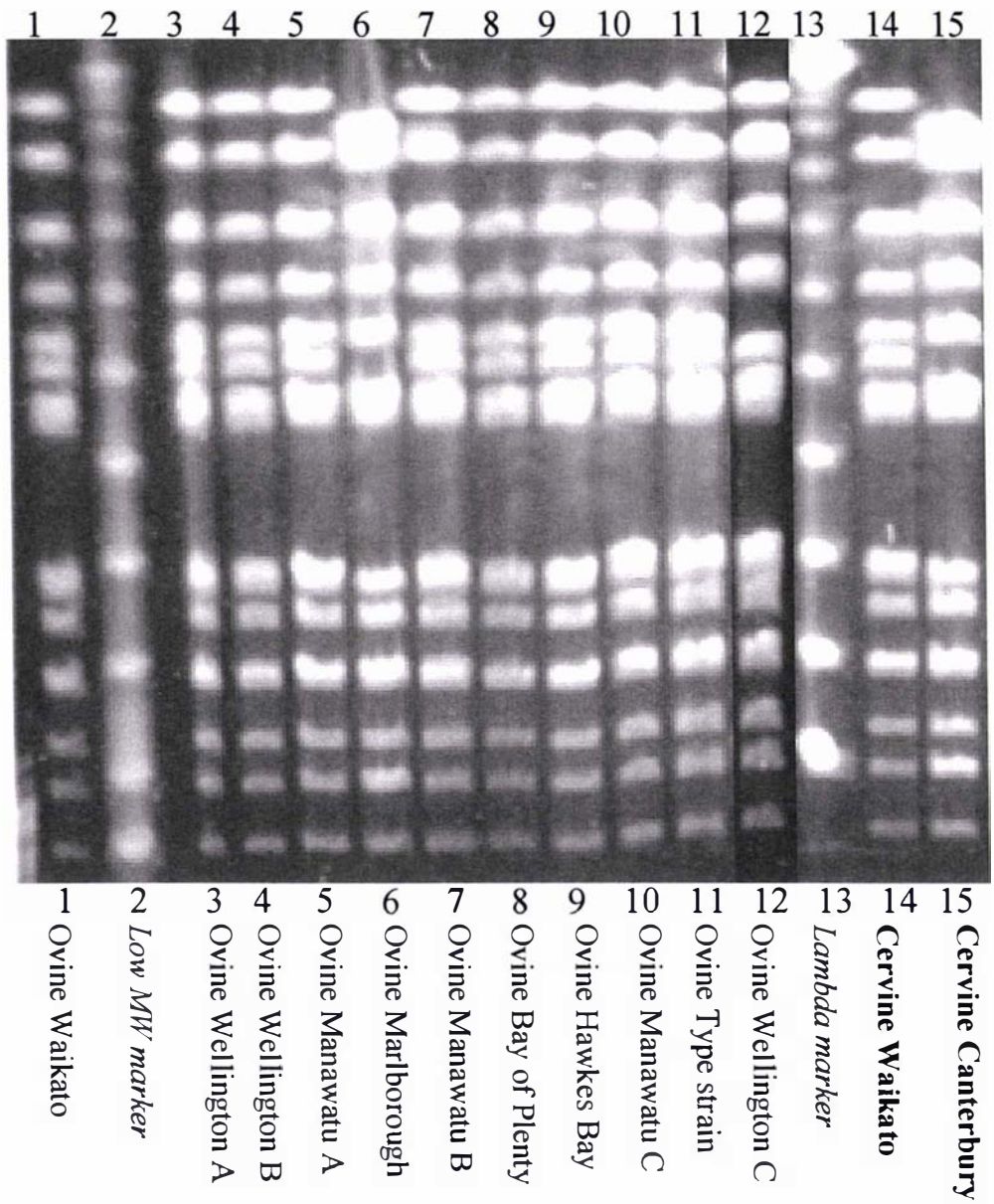


Figure 3.2 DNA band patterns resulting from pulsed field gel electrophoresis of *B. ovis* isolates from rams and stags in New Zealand. Ovine isolates are in normal text, cervine isolates are in bold text.

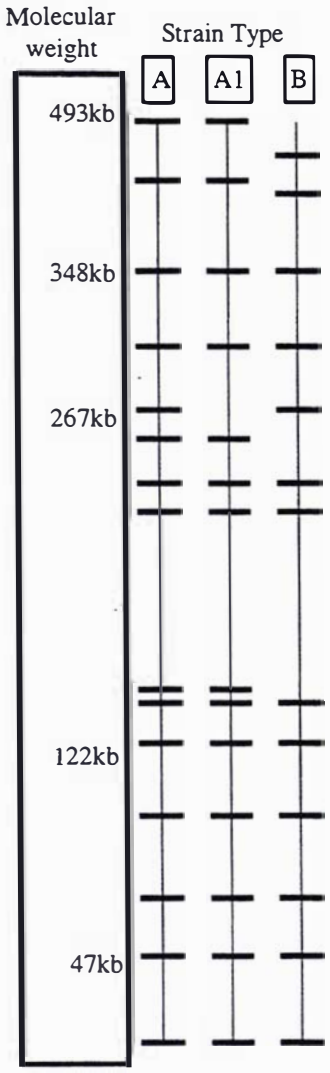


Figure 3.3 Schematic diagram of DNA bands of *B. ovis* isolates resulting from pulsed field gel electrophoresis, demonstrating three different banding patterns arbitrarily named Type A, Type A1 and Type B.

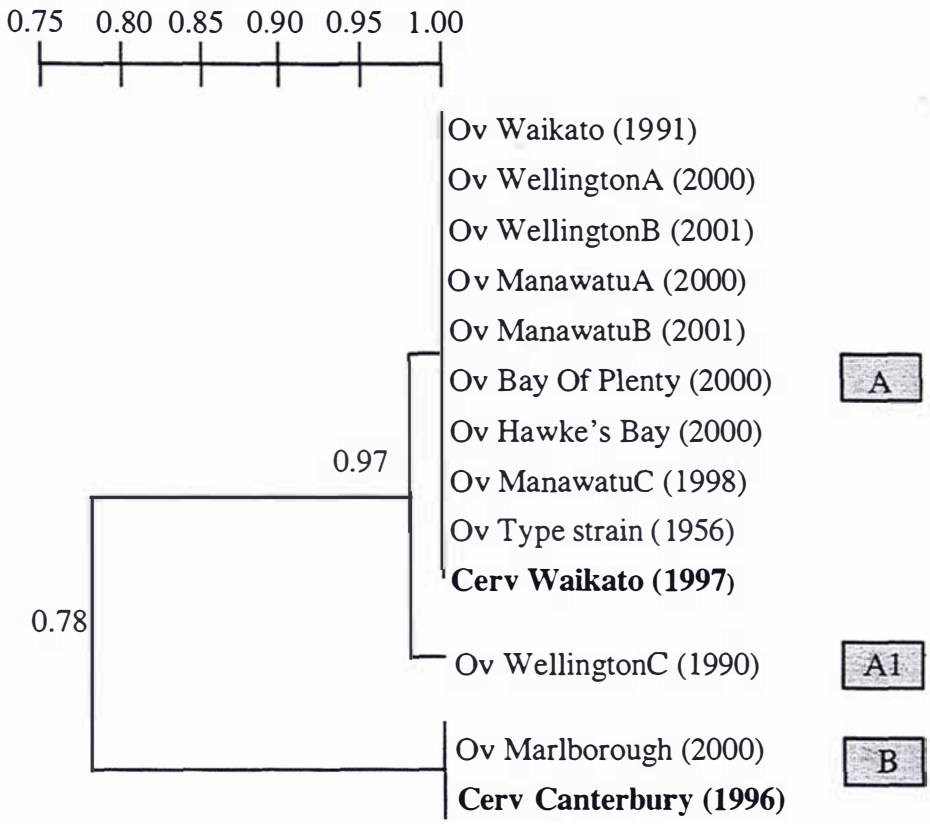


Figure 3.4 Schematic diagram (dendrogram) outlining the degree of similarity between *B. ovis* isolates from rams and deer throughout New Zealand following pulsed field gel electrophoresis. Ovine isolates are in normal text, cervine isolates are in bold text.

3.4 Discussion

This is the first report of convincing evidence of different strains of *B. ovis*. O'Hara et al. (1985), Bailey and West (1987) and Halling and Zehr (1990), failed to find different strains by analysis of *B. ovis* isolates using either Restriction Endonuclease analysis or Southern blotting of highly repeated DNA. Tcherneva et al. (2000), found some band differences when undertaking random amplification of polymorphic DNA (RAPD) analysis of four Belgium *B. ovis* isolates and the Type strain. However, a dendrogram analysis was not undertaken so the degree of similarity between the isolates was not stated. Also, RAPD analysis has been shown to have poor reproducibility (Meunier and Grimont, 1993; Tyler et al., 1997).

In previous attempts at strain typing *B. ovis*, only a small number of isolates were analysed. O'Hara et al. (1985) analysed 33 field strains but these isolates came from only six different farms. Bailey and West (1987) compared four field isolates, the Type strain and two vaccine strains while Halling and Zehr (1990) compared 12 field isolates from Australia and New Zealand. Similarly in this experiment only 13 isolates were analysed but different strains were identified. This may have been due to the different endonuclease used to cut the *B. ovis* DNA, and the use of pulsed field gel electrophoresis compared with others methods of analysis.

The *B. ovis* Type strain, which was isolated in 1956, showed an identical banding pattern to North Island *B. ovis* isolates which were collected in 2001, some 45 years later. This suggests that *B. ovis* is a very stable organism with little genetic drift over time.

In New Zealand, *B. ovis* infection of stags has been confirmed by bacteriological culture on only five properties. Two of these isolates were analysed in this experiment. One isolate was isolated in Canterbury in 1996 from the first infected stag found in New Zealand (O'Neil, 1996) and the other was isolated in Waikato in 1997 from an outbreak of infection in young stags which was detected at slaughter (Scott, 1999). These two cervine isolates were different strains, demonstrating that the Waikato infection could

not have occurred due to transfer of stags from the infected Canterbury property. This supports the epidemiological investigation of Scott (1999), that found no evidence of contact between deer from the two properties and lends support to the theory that the source of infection for some stags is from contact with infected rams.

From this PFGE analysis of New Zealand *B. ovis* strains, it is interesting that the ram and stag isolates from the North Island and the Type strain are all Type A yet the isolates from the South Island were both Type B. Unfortunately only one isolate was obtained from a South Island ram and one from a South Island stag, and therefore it is difficult to interpret this result. It would be useful to analyse further *B. ovis* isolates originating from both Islands of New Zealand to determine the distribution of the two strains.

3.5 Conclusions

1) Following pulsed field gel electrophoresis of *B. ovis* isolates, two strain types of *B. ovis* have been identified in the New Zealand ram and stag population. These strain types have been arbitrarily named Type A and Type B. A subtype of Type A, Type A1, was also identified.

2) *B. ovis* Types A and B were isolated from both rams and stags in New Zealand, lending support to the theory that the initial source of infection for stags in New Zealand was from infected rams.

Chapter Four

Transmission of *Brucella ovis* infection

A summary of Section 4.2, entitled "Transmission of *Brucella ovis* from rams to red deer stags" by AL Ridler, DM West, KJ Stafford, PR Wilson and SG Fenwick has been published in the New Zealand Veterinary Journal 48, 57-59, 2000.

A summary of Section 4.3, entitled "Attempted transmission of *Brucella ovis* between red deer stags by successive grazing or adjacent-paddock grazing" by AL Ridler, DM West, KJ Stafford, PR Wilson and SG Fenwick has been published in the New Zealand Veterinary Journal 48, 125-128, 2000.

4.1 Introduction

Brucella ovis infection of deer was recognised in the United States of America in 1984 (Barron, 1984) and in New Zealand in 1996 (O'Neil, 1996) but the source of infection was not determined. In both instances it was speculated that the source of infection was most likely to have been from sheep as they are the only known natural host for *B. ovis* (Barron, 1984; Scott, 1999).

In an experiment in which infected stags were grazed with non-infected rams and stags, there was no transmission of infection to the rams but there was transmission to the stags grazing in the same paddock (West et al., 1999). The mechanism by which infection was transmitted between the stags is unknown and it could not be determined whether transmission was by homosexual activity, as has been suggested for rams (Hartley, 1955), or by other mechanisms such as sniffing infected semen or by aerosol spray of infected semen. Furthermore, it was not known whether transmission of *B. ovis* between stags can occur by grazing contaminated pasture or whether stags grazing in a paddock adjacent to infected stags can become infected. These aspects are of practical significance for the successful control and management of *B. ovis* infected deer herds.

The objectives of this series of experiments were to investigate whether transmission of *B. ovis* infection can occur from rams to stags or between stags when they are not in direct contact with one another, and possible mechanisms of transmission.

4.2 Transmission of *B. ovis* from rams to stags

4.2.1 Introduction

While goats and laboratory animals have been successfully infected experimentally with *B. ovis* (Burgess et al., 1985; Cuba-Caparo and Myers, 1973) and there has been a report of infection occurring naturally in deer (Barron, 1984), infection is generally associated only with sheep. Despite trace-back studies being undertaken on three commercial New Zealand deer farms from which stags were found to be infected with *B. ovis* (Scott,

1998; Scott, 1999), the source of the infection for these stags was not determined. However, it seems probable that in at least some cases the infection may have come from infected rams.

The objective of this experiment was to graze infected rams in the same paddock as stags to determine whether transmission of *B. ovis* can occur from rams to stags.

4.2.2 Materials and methods

General sample collection and analysis methods are included in Chapter 2. A timeline of events is summarised in Figure 4.2.1.

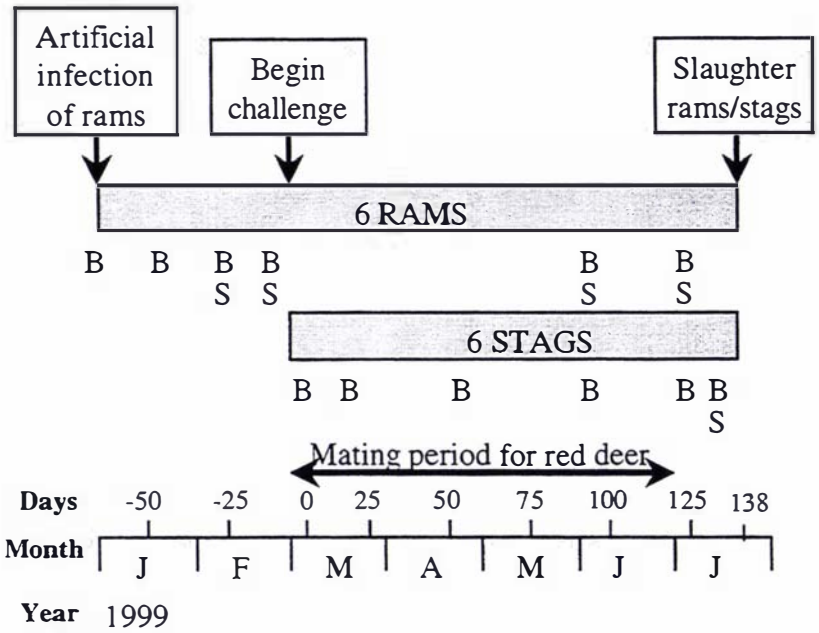


Figure 4.2.1 Timeline of events for an experiment investigating the transmission of *B. ovis* from infected rams to non-infected stags. B = blood samples collected, S = semen samples collected.

Animals

Six initially seronegative three-year-old Perendale rams and six 14-month-old red deer stags were used.

Artificial infection of rams

On January 14 1999, 50 days prior to the challenge period, the rams were artificially infected with *B. ovis* by intravenous injection of 2 ml of an inoculum containing 1.82x 10^9 colony forming units/ml into the jugular vein. The challenge period began on Day 0 when the rams were mixed with six non-infected stags. On Days -36, -23, -7, 92 and 124, blood samples were collected from the rams and tested for *B. ovis* antibodies using the CFT and ELISA. On Days -23, -7, and 92 semen samples were collected from the rams for *B. ovis* culture. On Day 92, immediately prior to semen collection, a sterile cotton swab was wiped inside the prepuce of each ram for *B. ovis* culture.

Challenge period

On Day 0 (March 4, 1999) the infected rams were shifted onto a two hectare paddock with the six stags. The rams and stags were grazed together in this paddock for 62 days. The animals were moved to a 0.6 hectare paddock from Days 62 to 92, then to a 0.5 hectare paddock from Days 92 to 123, and finally to another 0.5 hectare paddock from Day 123 until the stags were slaughtered on Day 138 (July 20, 1999). The animals were shifted into different paddocks according to feed availability.

Sampling of stags

Blood samples were collected from the stags on Days 20, 55, 92, 124 and 131 and tested for *B. ovis* antibodies testing using the CFT. On Day 131 semen samples were collected from the stags for *B. ovis* culture. On Day 138 the stags were slaughtered and the epididymes, seminal vesicles and ampullae were collected for gross pathological and histopathological examination, and for *B. ovis* culture.

4.2.3 Results

Ram infection following intravenous inoculation

Sera from all six experimentally infected rams became positive in the *B. ovis* CFT and ELISA within 14 days of artificial infection and serological titres remained in the positive range throughout the experiment (Figures 4.2.2, 4.2.3). Raw serological data is included in Appendix 1 (Table A1.1). *Brucella ovis* was cultured from the semen of five of the six rams collected on Days -23 and -7 prior to the challenge period and on Day 92 towards the end of the challenge period, but not from the semen of the sixth ram at any stage. *Brucella ovis* was cultured from the prepuce of four of the six rams on Day 92 (Table 4.2.1).

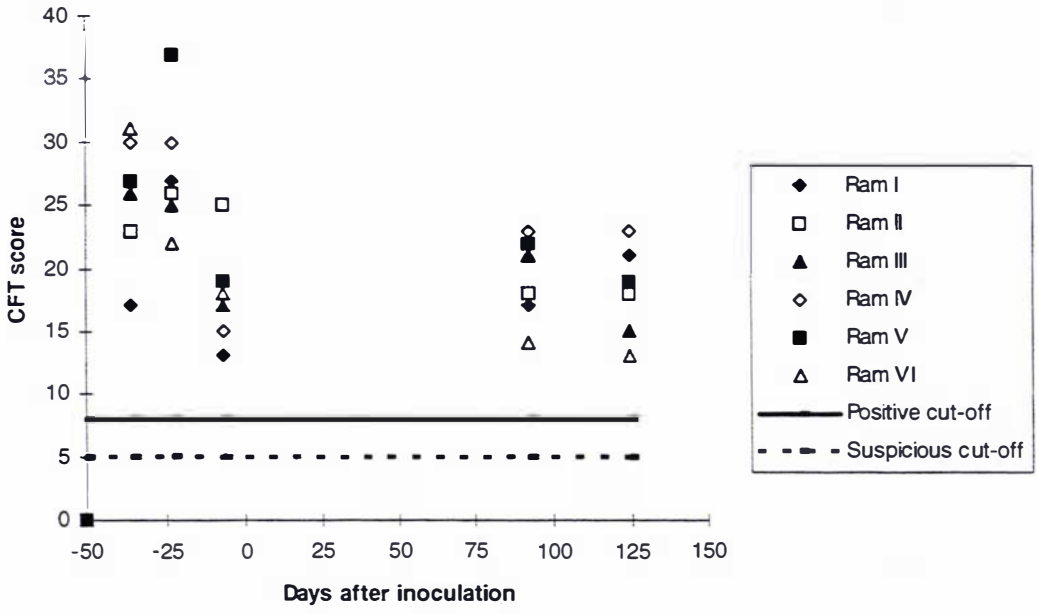


Figure 4.2.2 *B. ovis* CFT scores from sera collected from six rams following intravenous inoculation on Day -50.

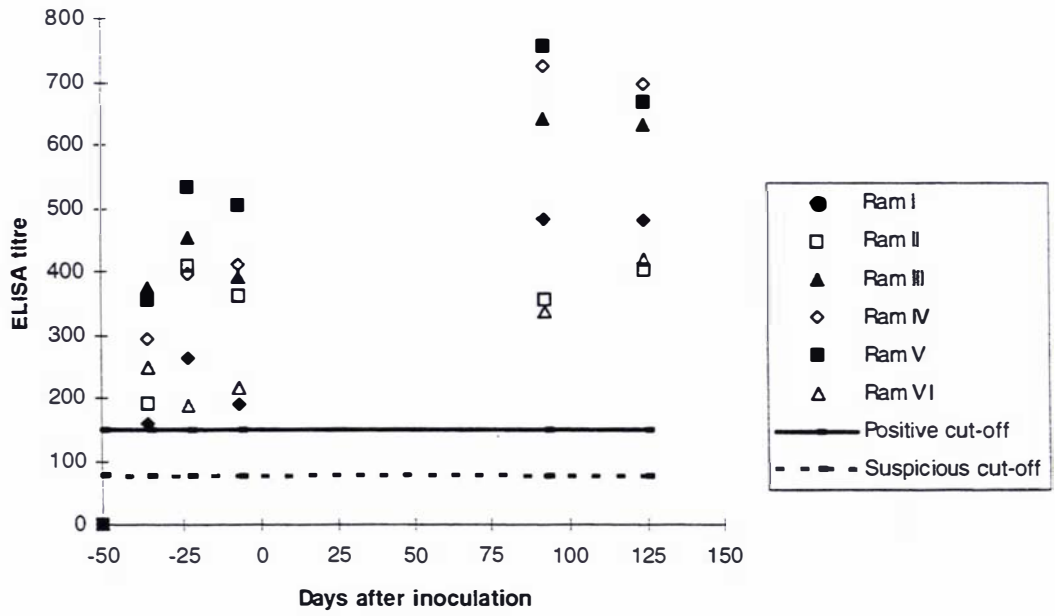


Figure 4.2.3 *B. ovis* ELISA tires from sera collected from six rams following intravenous inoculation on Day -50.

Table 4.2.1 *B. ovis* culture from the semen and prepuce of six experimentally infected rams following intravenous inoculation on day -50.

Ram ID	Semen Day -23	Semen Day -7	Semen Day 92	Prepuce Day 92
I	+	+	+	+
II	+	+	+	+
III	+	+	+	+
IV	+	+	+	+
V	+	+	+	-
VI	-	-	-	-

+ : *B. ovis* positive
- : *B. ovis* negative

Stag infection

Sera collected from the stags on Days 20, 55 and 92 were negative in the *B. ovis* CFT. On days 124 and 131 sera from five of the six stags were positive in the *B. ovis* CFT (Figure 4.2.4). Raw data is included in Appendix 1 (Table A1.2). On day 131, semen samples were successfully collected from four of the five seropositive stags and *B. ovis* was cultured from all four semen samples. *Brucella ovis* was cultured from the reproductive organs of all five seropositive stags when collected at slaughter on day 138. *Brucella ovis* was not cultured from the semen sample collected from the seronegative stag (stag number 6) on day 131, or from the reproductive organs collected from this stag at slaughter (Table 4.2.2).

Pulsed-field gel electrophoresis using the method described in Chapter 3 confirmed that the *B. ovis* isolates from these five stags were the same strain that had been used to artificially infect the six rams (Figure 4.2.5).

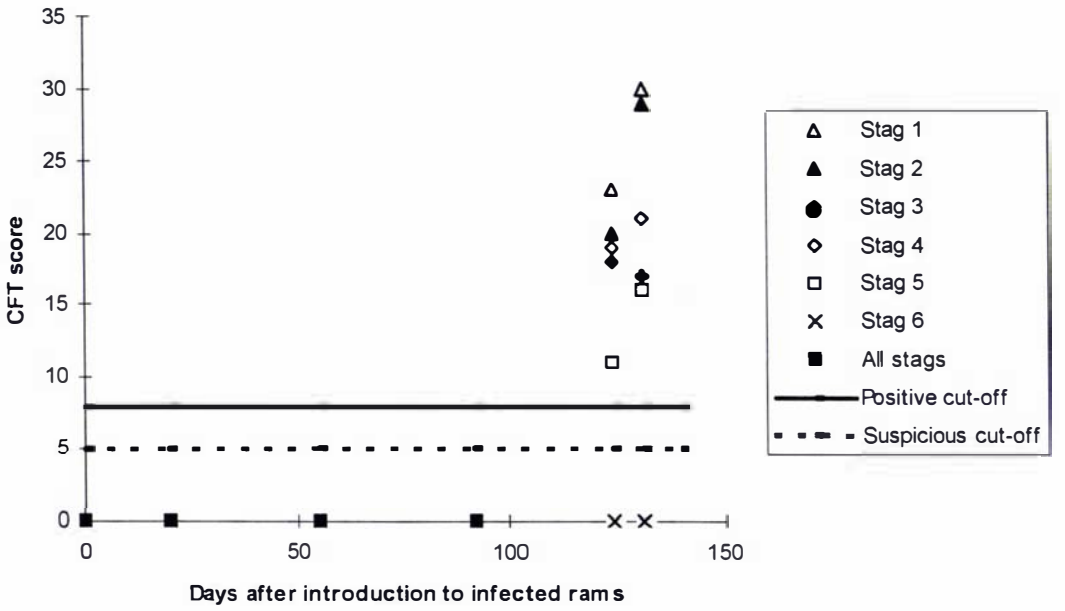


Figure 4.2.4 *B. ovis* CFT scores from sera collected from six stags following introduction to six infected rams on Day 0.

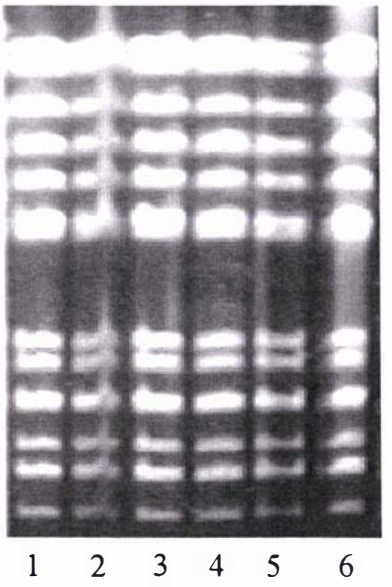


Figure 4.2.5 Pulsed-field gel electrophoresis of *B. ovis* isolates from five stags (Lanes 2-6) that became infected with *B. ovis* by contact with infected rams intravenously inoculated with the isolate in Lane 1.

Table 4.2.2 *B. ovis* culture from the semen and reproductive organs of six stags following introduction to six infected rams on Day 0.

Stag ID	Semen Day 131	Epididymes Day 138	Seminal vesicles Day 138	Ampullae Day 138
1	NS	+	+	+
2	+	-	+	-
3	+	-	+	+
4	+	-	+	+
5	+	+	+	+
6	-	-	-	-

+ : *B. ovis* positive
- : *B. ovis* negative
NS: no sample

Gross pathology

On Day 131, Stag 1 had lesions of epididymitis that could be detected by scrotal palpation and at necropsy on day 138 the tails of both epididymes from this stag were enlarged (Figure 4.2.6). On incision, these organs did not appear visibly abnormal. The tails of the epididymes from Stags 2 and 4 felt normal on scrotal palpation and at necropsy they appeared normal on external examination, but on incision were found to contain multiple three to four millimeter diameter foci of white caseous material (Figure 4.2.7). The epididymes of the remaining stags felt normal during scrotal palpation and at necropsy they appeared grossly normal. The seminal vesicles and ampullae of all stags appeared grossly normal.

Histopathology

Stags 1-5 had histological lesions in the epididymes, seminal vesicles and ampullae. Lesions consisted of lymphocytic infiltration into the lamina propria, in degree from small aggregates of lymphocytes to prominant lymphocytic follicles (Figures 4.2.8 and 4.2.9). The epididymes contained spermatic granulomas and occasional intra-epithelial cysts (Figures 4.2.10 and 4.2.11). The ducts within the seminal vesicles and ampullae contained neutrophils and lymphocytes. There were no histological lesions in the epididymes, seminal vesicles or ampullae from stag 6.

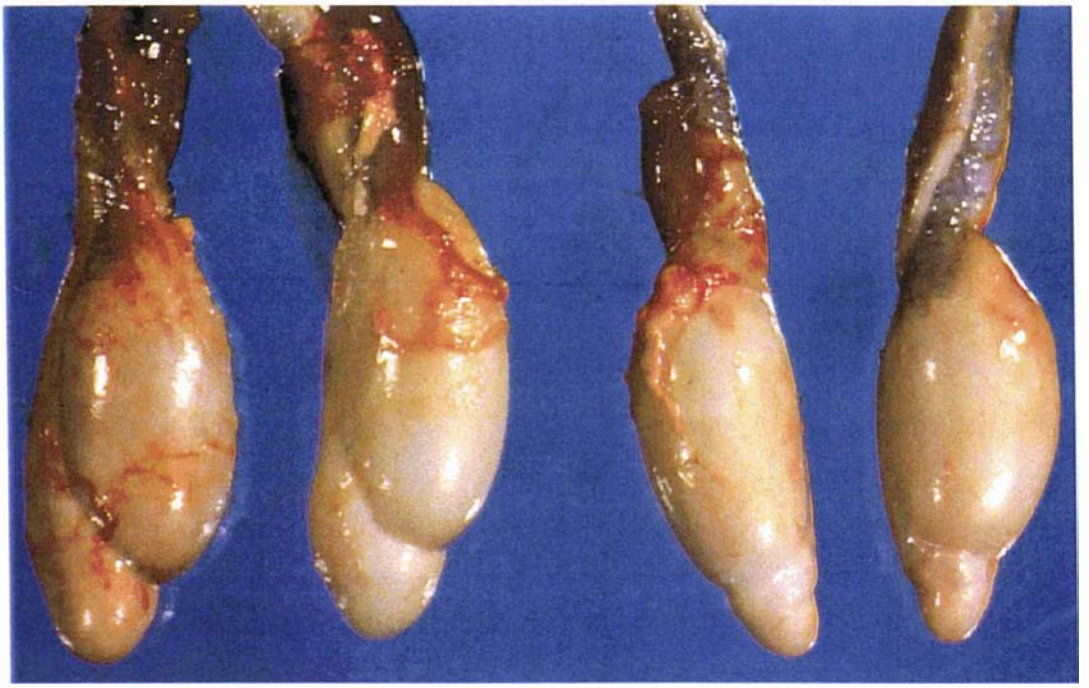


Figure 4.2.6 Bilateral enlargement of the tails of the epididymes of stag 1 (left) compared with normal tails of the epididymes of stag 5 (right).

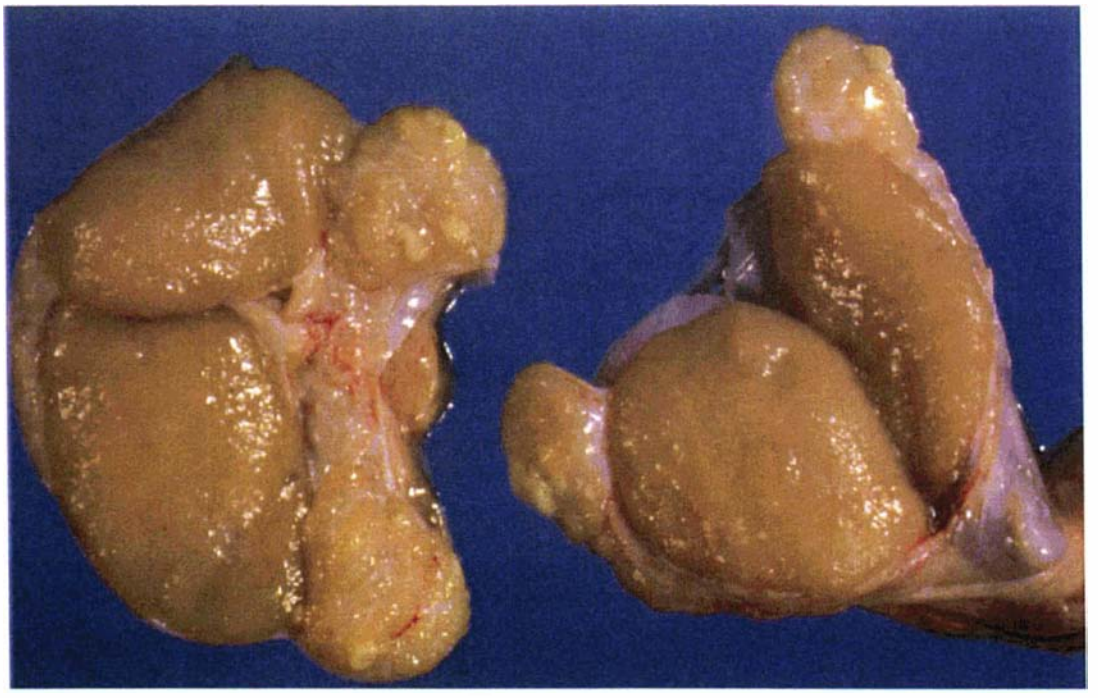


Figure 4.2.7 Foci of caseous material within the tails of the epididymes from stag 2.

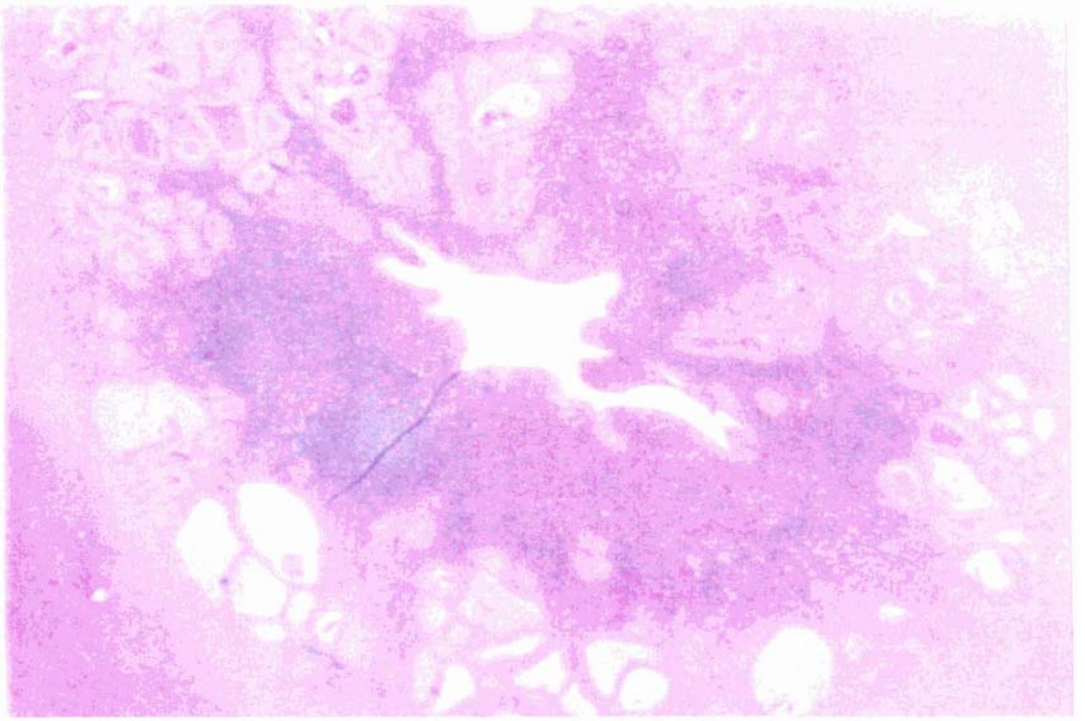


Figure 4.2.8 Prominent lymphocytic infiltration into the lamina propria of the ampulla of stag 1.

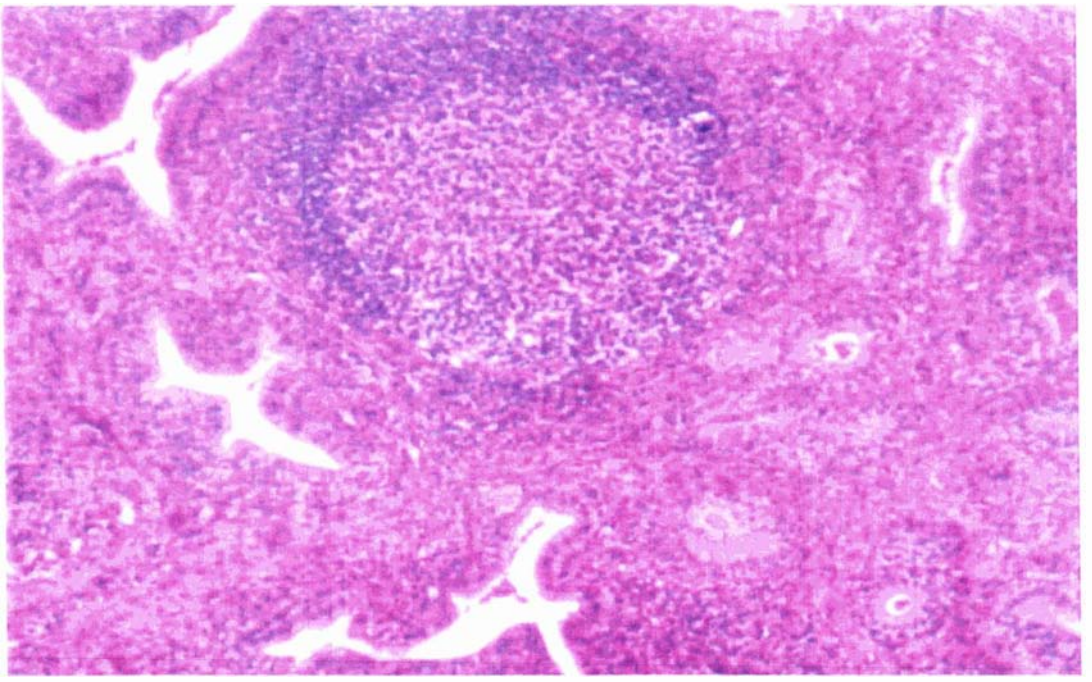


Figure 4.2.9 Generalised lymphocytic infiltration and a lymphoid follicle in the lamina propria of the seminal vesicle from stag 4.

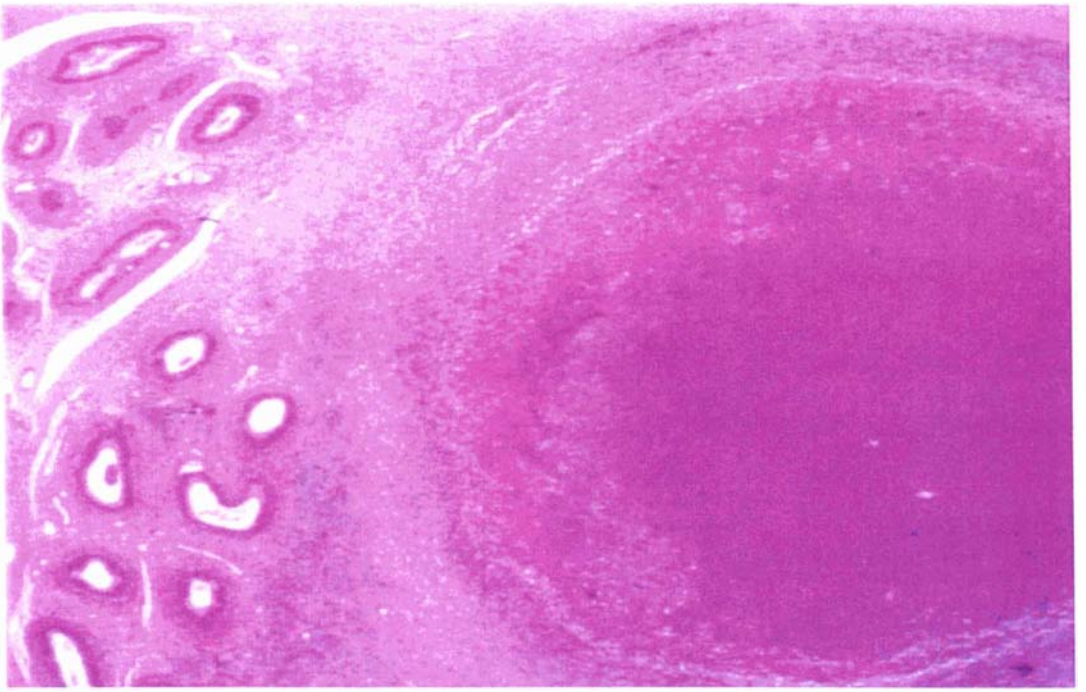


Figure 4.2.10 A spermatic granuloma in the tail of the left epididymis of stag 2.

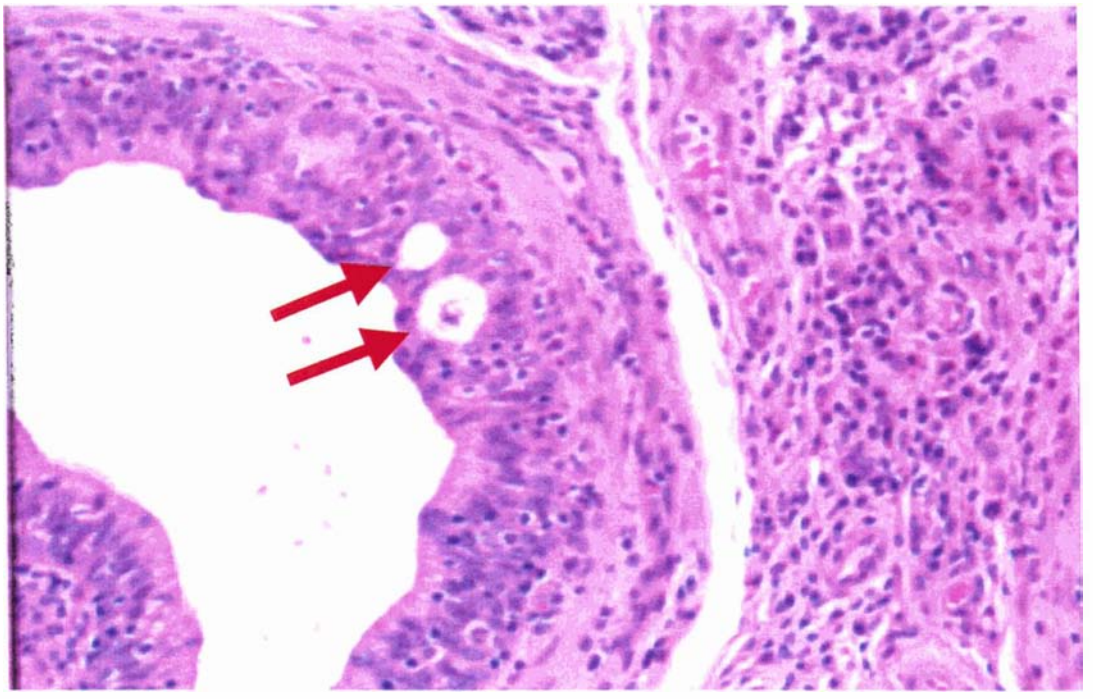


Figure 4.2.11 Intra-epithelial cysts (red arrows) in the tail of the right epididymis of stag 4

4.2.4 Discussion

This experiment demonstrated transmission of *B. ovis* from infected rams to five of six stags grazing in the same paddock. Epidemiological investigations reported by Scott (1999) from three commercial farms on which stags were found to be infected revealed that there were no links between the properties in terms of deer introductions from the same source. The properties were in different geographical locations suggesting that infection from deer straying from one property to the next was highly unlikely (Scott, 1999). Knowledge from the present study that transmission can occur from rams to stags suggests that the source of infection in at least some of these cases is likely to have been from stag contact with infected rams.

In July 2000, a veterinarian (J. Smart, *pers. comm.*) investigating *B. ovis* infection in a ram flock on a South Island sheep, beef and deer property also collected blood from 12 mixed-age stags that had been in contact with the infected rams. Sera from three of the stags were positive or suspicious in the *B. ovis* CFT and these three stags were culled but bacteriological culture of semen or the reproductive tract was not attempted. There had been no recent introductions of deer into this herd and the probable stag infections were almost certainly due to contact with the infected rams. The actual prevalence of *B. ovis* in the New Zealand sheep population is unknown but in 1996, 1.1% of stud rams and 5.6% of commercial rams tested for the *B. ovis* voluntary accreditation scheme were seropositive (Reichel and West, 1997). It is common for sheep and deer to be farmed on the same property and therefore there is potential for sporadic infection to occur in deer due to contact with infected rams.

The pattern of infection in the stags is considered significant. Up until 92 days after mixing there was no serological evidence of infection in the stags but when sampled 32 days later on day 124, five of the six stags had high serum antibody levels in the CFT. This suggests that all five stags became infected at a similar time, possibly from the same source, rather than one stag becoming infected and then spreading infection to other stags. It has been demonstrated in sheep that following experimental infection of rams via mucous membranes there is a minimum period of four weeks before the organism is shed in the semen (Table 1.3.1, Laws et al., 1972; Webb et al., 1980; Plant

et al., 1986). It is demonstrated in Section 4.4 that stags develop serum antibodies by at least 11 days after inoculation of mucous membranes with pure cultures of *B. ovis*. If the pathogenesis of infection in stags is similar to rams, 32 days is unlikely to be sufficient time for one stag to become infected, shed *B. ovis* in the semen, infect the other stags, and for those stags to seroconvert. Thus it seems likely that all five stags became infected at about the same time, probably from direct contact with infected rams. The most likely ways the stags became infected would be from sniffing the prepuce of infected rams, sniffing the perineal region of a ram after it had been mounted by another ram, or aerosol spray of infected semen contacting the conjunctiva or other mucous membranes. Alternatively it is possible that transmission occurred from sniffing infected semen or urine deposited on pasture.

While the organs from only five infected stags were examined in this experiment, it would appear that the seminal vesicles might be the site of choice for culture of *B. ovis* from necropsy specimens. *Brucella ovis* was cultured from the seminal vesicles of all five infected stags but was cultured from the epididymes of only two of the five infected stags, both of which had grossly visible epididymal lesions on incision of the organs. *Brucella ovis* was isolated from the ampullae of four of the five stags but due to the small diameter of this organ it is difficult to sample without contamination.

Histological examination of the epididymes, seminal vesicles and ampullae of acutely infected stags revealed similar lesions to those identified in naturally infected rams (Kennedy et al., 1956; Biberstein et al., 1964) and experimentally infected gerbils (Cuba-Caparo and Myers, 1973). These lesions consist of lymphocytic infiltration into the lamina propria of all three organs, and spermatic granulomas and intra-epithelial cysts in the epididymes. Barron et al. (1985) described similar lesions in the epididymes of artificially infected white-tailed deer bucks.

4.3 Transmission of *B. ovis* between stags by successive grazing or adjacent-paddock grazing

4.3.1 Introduction

It has been demonstrated that transmission of *B. ovis* can occur between stags when in direct contact with one another. Barron (1984) grazed an infected white-tailed deer buck with a non-infected buck for a period of 238 days. At the end of this time, the non-infected buck was slaughtered and found to have lesions of epididymitis from which *B. ovis* was cultured. West et al. (1999) grazed two infected rising-two-year-old stags with eight non-infected stags of the same age from September to June. Animals were blood sampled monthly and in May there was serological evidence that four of the eight control stags became infected. The timing of infection suggests that the rut period and sexual activity is important in the transmission of *B. ovis* between stags.

While these experiments have confirmed that transmission of *B. ovis* can occur between stags when they are in direct contact with each other, it is also important to establish whether transmission of infection can occur by "indirect" methods such as animals grazing in a paddock previously grazed by infected animals or by contact with infected animals through a fence. This information would assist in the management of infected deer herds by establishing the degree of separation required between infected and non-infected stags to reduce the risk of transmission between the two groups.

The objective of this experiment was to establish whether transmission of *B. ovis* between stags can occur by grazing paddocks previously grazed by infected stags (successive grazing) or by having through-the-fence contact with infected stags (adjacent-paddock grazing).

4.3.2 Materials and methods

General sample collection and analysis methods are described in Chapter 2. A timeline of events is summarised in Figure 4.3.1.

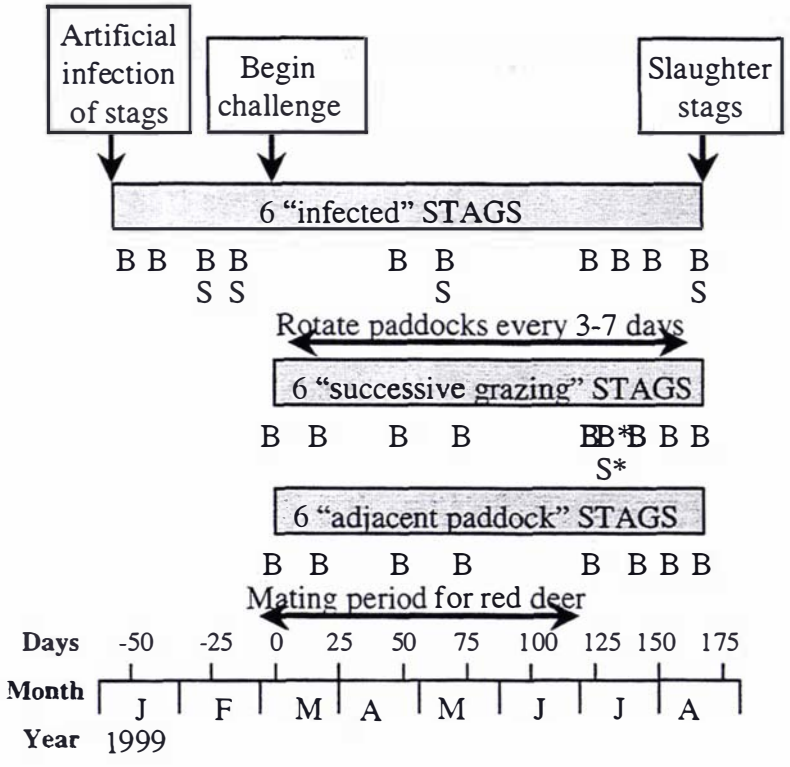


Figure 4.3.1 Timeline of events for an experiment investigating the transmission of *B. ovis* between stags by successive grazing or adjacent-paddock grazing. B = blood samples collected, S = semen samples collected, * = only 1 stag sampled.

Animals

Six 14-month-old red deer stags were artificially infected with *B. ovis* and these were used as the challenge source of infection. Twelve, initially seronegative, 14-month-old red stags were used to determine whether transmission of infection occurred by successive grazing or adjacent-paddock grazing.

Artificial infection of stags

On January 14 1999, 49 days prior to the challenge period, six stags were artificially infected with *B. ovis* by intravenous injection of 2 ml of an inoculum containing

1.82×10^9 colony forming units/ml. Blood samples were collected from infected stags on Days -49, -35, -22, -6, 56, 69, 124, 139, 154 and 168 for *B. ovis* antibody testing using the CFT and ELISA. On Days -22, -6, 69 and 168 semen samples were collected from the artificially infected stags for *B. ovis* culture.

Challenge by successive grazing or adjacent-paddock grazing

Between Days 0 and 168, six stags alternated between one-hectare paddocks with the group of infected stags (successive grazing). Every three to seven days the two groups alternated paddocks so that the non-infected stags were put into a paddock that had only just been vacated by infected stags. There was no direct physical contact between the groups. During the experiment, the two groups alternated paddocks a total of 32 times. A further six stags were grazed in a one-hectare paddock situated between the two paddocks grazed by the above two groups (Figure 4.3.2). Therefore these stags were grazing in a paddock adjacent to the infected stags throughout the five and a half month trial period, separated from them by a 1.8 metre high, 12-gauge wire-netting fence with netting grids of 165 mm by 175 mm. The netting was supported by round posts at five metre intervals (Figure 4.3.3).

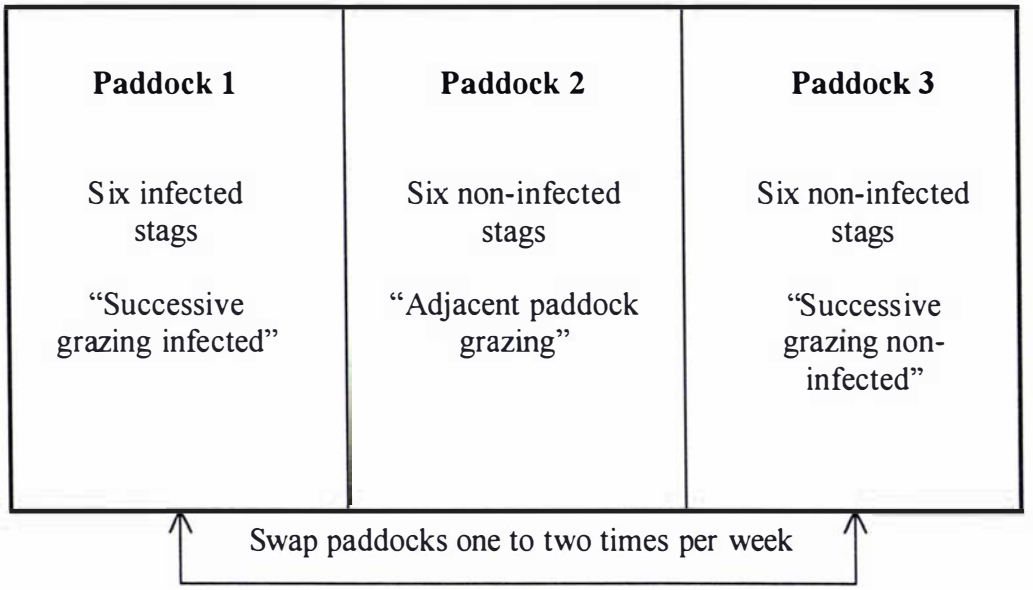


Figure 4.3.2 Schematic diagram of paddocks and deer groups used to determine transmission of *B. ovis* between stags by successive grazing of the same paddock, or by adjacent-paddock grazing. Each paddock was approximately one hectare in size.

Figure 4.3.3 Six stags artificially infected with *B. ovis* grazing in a paddock adjacent to six non-infected stags. The two groups are separated by a standard New Zealand deer fence that is 1.8m high, with 12-gauge wire-netting grids of 165 mm by 175 mm supported by round posts at 5m intervals.

Sampling of non-infected stags

Blood samples were collected from the stags in the successive grazing non-infected and the adjacent paddock grazing groups on Days 0, 21, 57, 69, 124, 139, 154 and 168 for testing in the CFT and ELISA. A further blood sample, and a semen sample were collected from one stag from the successive grazing non-infected group on day 131 for CFT and ELISA testing and semen culture. The epididymes, seminal vesicles and ampullae of this stag were collected at slaughter on day 168 for *B. ovis* culture.

4.3.3 Results

Stag infection following intravenous inoculation

Sera from all six experimentally infected stags became positive in the *B. ovis* CFT within 14 days of inoculation. The serological titres of five stags remained above the cut-off level for positive reactions until Day 124. After this time, sera from some stags gave suspicious or negative reactions in either the CFT or the ELISA (Figures 4.3.4, 4.3.5). Serum from the sixth stag initially reacted strongly in both the CFT and ELISA but became seronegative between 45 and 100 days after inoculation (Figures 4.3.4, 4.3.5). Raw serological data is included in Appendix 1 (Tables A1.3, A1.4).

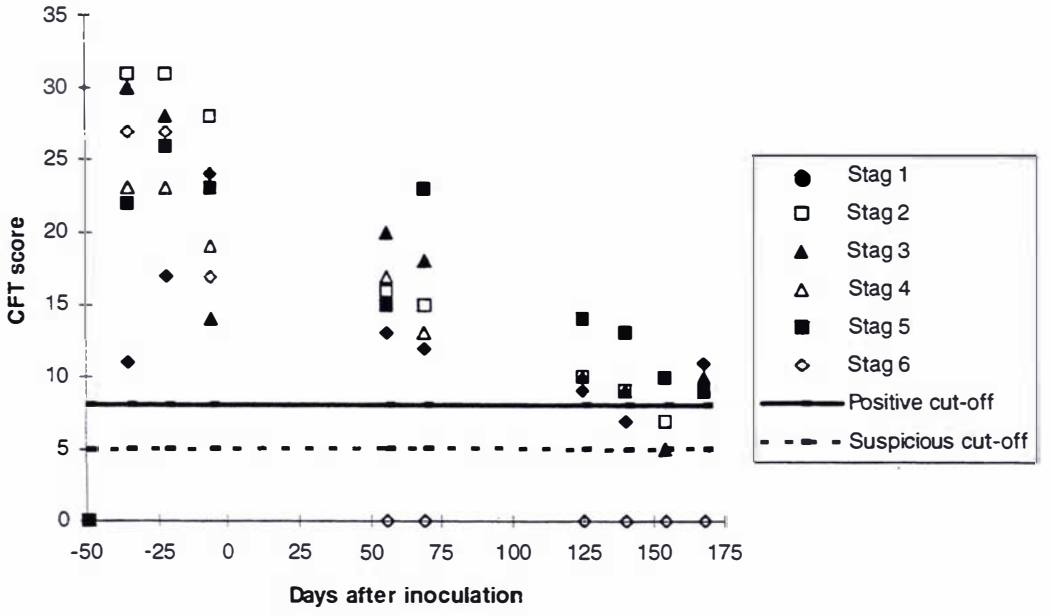


Figure 4.3.4 *B. ovis* CFT scores for six stags artificially infected by intravenous inoculation on Day -49.

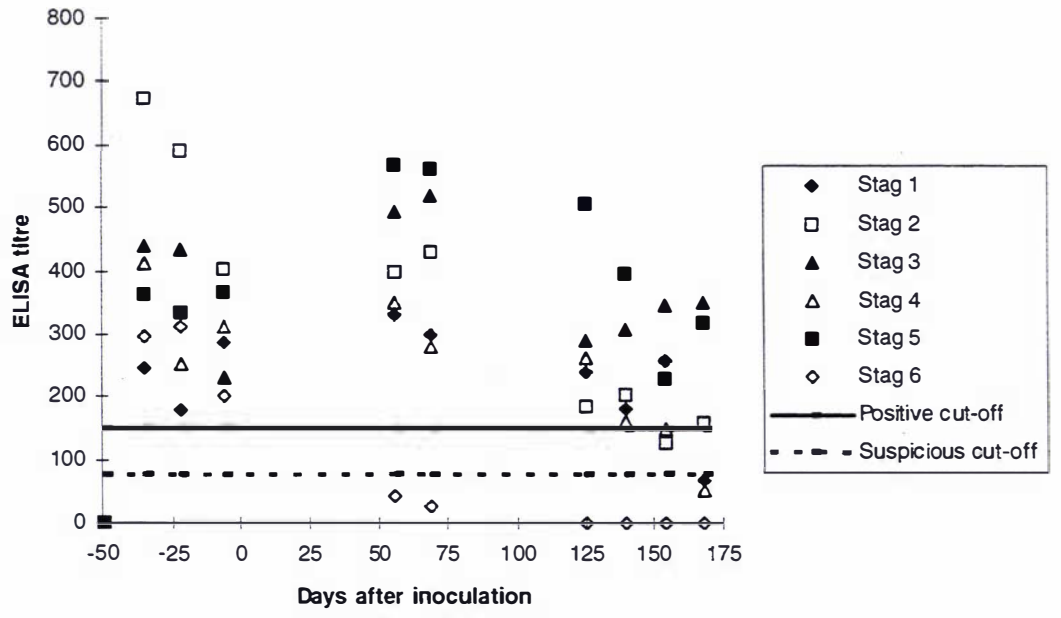


Figure 4.3.5 *B. ovis* ELISA titres for six stags artificially infected by intravenous inoculation on Day -49.

Brucella ovis was cultured from the semen of five of the six stags when sampled on days -22 and -6 prior to the challenge period and on days 69 and 168 during the challenge period but not from semen of the sixth stag at any stage (Table 4.3.1).

Table 4.3.1 *B. ovis* culture from the semen from six stags artificially infected by intravenous inoculation on Day –49.

Stag ID	Day -22	Day -6	Day 69	Day 168
1	+	NS	NS	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	-	-	-	-

+ : *B. ovis* positive
- :*B. ovis* negative
NS : no sample

Transmission of B. ovis between stags

On Day 124 of the challenge period, serum from a single stag in the successive grazing non-infected group had a CFT titre of 2/4 (negative) and an ELISA titre of 14 (negative). In all previous samplings, stags from the successive grazing non-infected and the adjacent paddock grazing groups had CFT titres of zero and it was decided to re-sample this stag. Seven days later, on Day 131, a further blood sample and a semen sample were collected from this stag. The serum was positive in the CFT with a titre of 1/32 and negative in the ELISA with a titre of 4. *Brucella ovis* was not cultured from semen. Serum from this stag was negative in the CFT and ELISA eight days later on Day 139 and in all subsequent tests, and *B. ovis* was not cultured from the epididymes, seminal vesicles or ampullae from this stag collected at slaughter on day 168.

Sera from other stags in both the successive grazing non-infected group and the adjacent paddock grazing group were not suspicious or positive in the *B. ovis* CFT or ELISA at any of the sampling times.

4.3.4 Discussion

This experiment demonstrated that transmission of *B. ovis* did not occur between stags by successive grazing or adjacent paddock grazing. The single positive CFT titre of 1/32 at testing on Day 131 from a stag in the successive grazing non-infected group is considered to be a false positive reaction because the same serum sample was negative in the ELISA, and when sampled eight days later on Day 139 the serum from this stag was negative in both the CFT and ELISA. It is unlikely that a positive titre in an infected stag would return to zero in this short period of time. In addition, *B. ovis* was not cultured from semen collected on Day 131 or from the epididymes, seminal vesicles or ampullae collected from this stag at slaughter on Day 168.

In experimental studies, rams have been artificially infected with *B. ovis* and developed serum antibodies but the organism has not been cultured from semen or reproductive organs, suggesting the infection did not localise in the reproductive tracts of these rams (Plant et al., 1986). Similar findings have occurred with artificially infected stags during the course of these experiments. Although it is possible that the single positive CFT titre from a stag in the successive grazing non-infected group may have been an antibody response due to exposure to *B. ovis*, the serological reactions of this stag suggest this is unlikely. Experimentally infected stags that did not shed *B. ovis* in semen initially had high serum antibody titres in both the CFT and ELISA and maintained these titres for at least 40 to 80 days (Section 4.4, Chapter 5). The stag from the successive grazing non-infected group had a single positive titre in the CFT only, and serum collected eight days later was negative in the CFT.

From this experiment it would appear that the risk of transmission of *B. ovis* to stags from successive grazing or adjacent-paddock grazing is low. While it is not possible to conclude that transmission of *B. ovis* will never occur by these means, the potential challenge presented to the control stags in this experiment was relatively high compared to what might occur during the control of infection on a commercial farm. Non-infected stags entered paddocks immediately vacated by infected stags on 32 occasions, or were paddocked next to infected stags for 168 days (five and a half months). The experimental period included the natural mating period of red deer, during which

transmission between stags has previously been demonstrated (West et al., 1999). This result is consistent with observations where transmission of *B. ovis* in sheep by successive grazing did not occur (Buddle, 1955; Hartley et al., 1955; Keogh et al., 1958) and rams grazing in paddocks adjacent to infected rams have not contracted *B. ovis* infection. Furthermore, practical experience gained from eradicating *B. ovis* from commercial ram flocks would suggest that physically separating infected and non-infected rams is sufficient to prevent transmission of *B. ovis*.

Therefore, it would appear that transmission of *B. ovis* occurs when animals are in direct contact with each other in the same paddock or pen. This has important implications for the control of *B. ovis* on a property. Keogh et al. (1958) demonstrated that infected and non-infected sheep flocks could be maintained on the same property without cross-infection occurring provided animals were not mixed, and it is likely that this also applies to the management of the disease in deer.

4.4 Routes of transmission of *B. ovis* between stags

4.4.1 Introduction

Experimentally, rams have been successfully infected with *B. ovis* by instillation of infected semen or a culture of *B. ovis* onto the nasal, rectal, preputial (Plant et al., 1986) and conjunctival mucous membranes (Biberstein et al., 1964; Brown et al., 1973). Inoculation of the oral mucous membranes of two rams resulted in histological lesions in the reproductive tract suggestive of infection in one ram (Simmons and Hall, 1953). Following inoculation of *B. ovis* onto mucous membranes of rams it appears that the organism localises in the regional lymph nodes before undergoing a bacteraemia and localising in the reproductive tract and, sometimes, other body organs (Biberstein et al., 1964). In white-tailed deer, Barron et al. (1985) was able to demonstrate successful infection of bucks by conjunctival instillation of a *B. ovis* culture but other routes for infection in this species are unknown.

The objective of this experiment was to determine whether stags can become infected with *B. ovis* by the instillation of a *B. ovis* culture onto the nasal, oral, conjunctival or rectal mucosa.

4.4.2 Materials and methods

General sample collection and analysis methods are described in Chapter 2.

Animals

Sixteen initially seronegative 10-month-old red deer stags were used.

Infection of stags

On October 9 2000 (Day 0) the 16 stags were ranked according to body weight and assigned to four groups of four animals each by simple random allocation depending on bodyweight ranking. Using a 3ml sterile syringe, each group was artificially infected with 1ml of an inoculum containing $3x10^9$ colony forming units/ml of *B. ovis* using the following methods:

- Conjunctival group; Stags C1-4 – inoculation was achieved by applying inoculum under the ventral eyelid of the right eye
- Nasal group; Stags N1-4 – inoculation was achieved by lifting the head and applying inoculum 1cm into the right nostril. The head was kept raised for approximately 30 seconds after instillation
- Oral group; Stags O1-4 – inoculation was achieved by inserting the syringe 1 cm into the mouth caudal to the incisor teeth and applying the inoculum
- Rectal group; Stags R1-4 – inoculation was achieved by inserting the syringe 1.5cm into the rectum and applying the inoculum

Blood samples were collected from stags on Days 0, 11, 24, 36, 56 and 65 for testing in the CFT and ELISA, and semen was collected on Day 56 for culture for *B. ovis*. Stags were slaughtered on December 13 2000 (day 65) and the epididymes, seminal vesicles and ampullae were collected for gross and histopathological examination, and for *B. ovis* culture.

4.4.3 Results

Conjunctival inoculation

Sera from all four stags in this group were positive in the CFT on Day 11 and remained positive until Day 36, but titres had declined by Day 56. In the ELISA, sera from these stags were negative or suspicious on Day 11, positive or suspicious on Days 24, 36 and 56 and negative or suspicious on Day 65 (Figures 4.4.1, 4.4.2). Raw serological data is included in Appendix 1 (Table A1.5).

Brucella ovis was cultured from the semen collected from stag C1 on Day 56 and from the epididymes of this stag collected at slaughter on Day 65 (Table 4.4.1) but at slaughter serum from this stag was negative in both the ELISA and CFT. Gross lesions were not detected in the epididymes, seminal vesicles and ampullae but histological examination of these organs revealed spermatic granulomas and intra-epithelial cysts in the epididymes, and lymphoid foci in the ampullae consistent with *B. ovis* infection. No histological lesions were detected in the seminal vesicles (Table 4.4.2).

Brucella ovis was not cultured from the semen or reproductive organs of stags C2, C3 or C4 (Table 4.4.1). There were no gross lesions in the reproductive tracts but on histological examination, stag C2 had small (10 to 20 cell) lymphoid foci in the epididymes and ampullae and stag C3 had small lymphoid foci in the epididymis, seminal vesicles and ampullae consistent with *B. ovis* infection. Stag C4 had a single lymphoid focus in one ampulla but no other lesions consistent with *B. ovis* infection. The significance of this lesion was difficult to assess (Table 4.4.2)

Nasal inoculation

Sera from the nasal inoculated stags were positive in the CFT on Days 11, 24 and 36 but titres had declined by Day 56. In the ELISA, sera from these stags were negative or suspicious at all sampling times with the exception of Day 36, when sera from stags N1, N3 and N4 were positive and serum from stag N2 was suspicious (Figures 4.4.1, 4.4.2). Raw serological data is included in Appendix 1 (Table A1.5).

Brucella ovis was cultured from the semen of stag N2 collected on Day 56 and from the epididymes and seminal vesicles collected at slaughter on Day 65 (Table 4.4.1) although at slaughter serum from this stag was only suspicious in the CFT and negative in the ELISA. On gross examination this stag had a subtle increase in the size of the tail of the left epididymis and histological examination revealed mild lymphoid foci in the epididymes and ampullae consistent with *B. ovis* infection (Table 4.4.2).

Brucella ovis was not cultured from the semen or reproductive tracts of stags N1, N3 or N4 (Table 4.4.1). At slaughter on Day 65, no gross lesions were detected in the reproductive tracts but on histological examination, stag N4 had very mild lymphoid foci in the ampullae and intra-epithelial cysts in the epididymes suggestive of *B. ovis* infection. There was no evidence of histological lesions in the epididymes, seminal vesicles and ampullae of stags N1 or N3 (Table 4.4.2).

Oral inoculation

On Day 11, sera from all four stags were negative in both the CFT and ELISA. In the CFT, sera from three stags were positive or suspicious on Days 24 and 36 but negative on Days 56 and 65. All sera remained negative in the ELISA on all sampling days with the exception of Day 36, when sera from two stags was in the suspicious range (Figures 4.4.1, 4.4.2). Raw serological data is included in Appendix 1 (Table A1.5).

Brucella ovis was not cultured from the semen or reproductive organs of any of the stags (Table 4.4.1). No gross lesions were detected in the reproductive tract of any of the stags but histological examination revealed occasional small lymphoid foci in the epididymes and seminal vesicles of stag O4 suggestive of *B. ovis* infection. No histological lesions were detected in the epididymes, seminal vesicles or ampullae of stags O1-3 (Table 4.4.2).

Rectal inoculation

Sera from all four rectal inoculated stags were positive or suspicious in the CFT at all sampling times after inoculation (Figure 4.4.1). In the ELISA, sera were negative or suspicious on Day 11 and sera from stags R2 and R4 were suspicious on Days 24 and 65 respectively but otherwise sera from all four stags were positive at all sampling times

after inoculation (Figure 4.4.2). Raw serological data is included in Appendix 1 (Table A1.5).

Brucella ovis was cultured from the semen of stags R1 and R2 and R4 on Day 56 and from the reproductive tract of stags R1 and R2 at slaughter on Day 65 (Table 4.4.1). On gross examination, stag R2 had a subtle increase in the size of the tail of the right epididymis but no gross lesions were present in the reproductive tracts of the other three stags. Stag R1 had histological lesions of lymphoid foci in the epididymes and ampullae, and stags R2 and R4 had lymphoid foci in the epididymes, seminal vesicles and ampullae, and intra-epithelial cysts in the epididymes consistent with *B. ovis* infection.

Brucella ovis was not cultured from the semen or reproductive tract from stag R3. There were no gross lesions in the reproductive tract but lymphoid foci were present in the epididymes, seminal vesicles and ampullae, and intra-epithelial cysts were present in the epididymes, consistent with *B. ovis* infection.

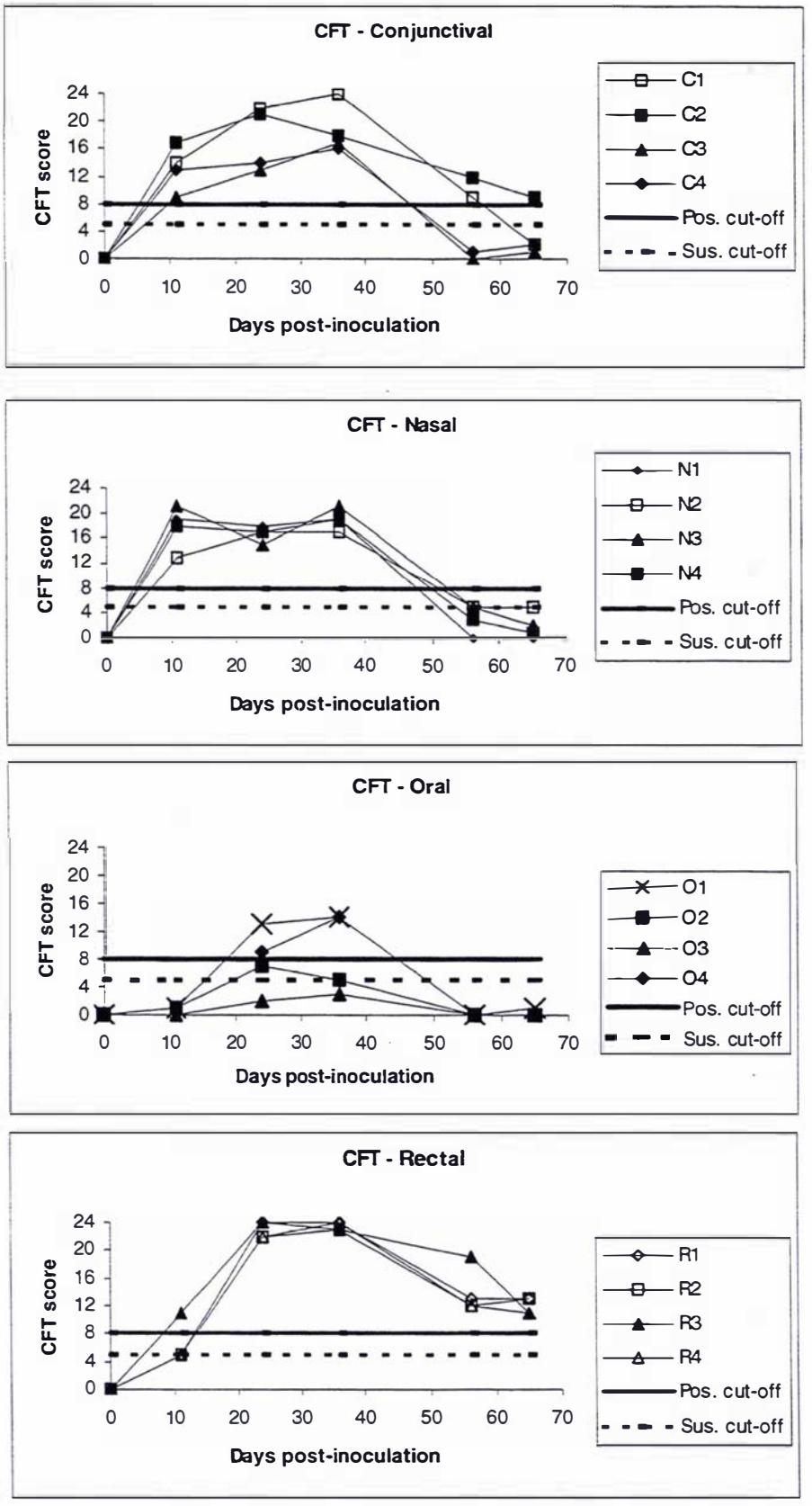


Figure 4.4.1 *B. ovis* CFT scores for yearling stags artificially infected with *B. ovis* by inoculation of either the conjunctival, nasal, oral or rectal mucous membranes. Stags from which *B. ovis* was isolated from either semen or the reproductive tract at slaughter are denoted by an open symbol.

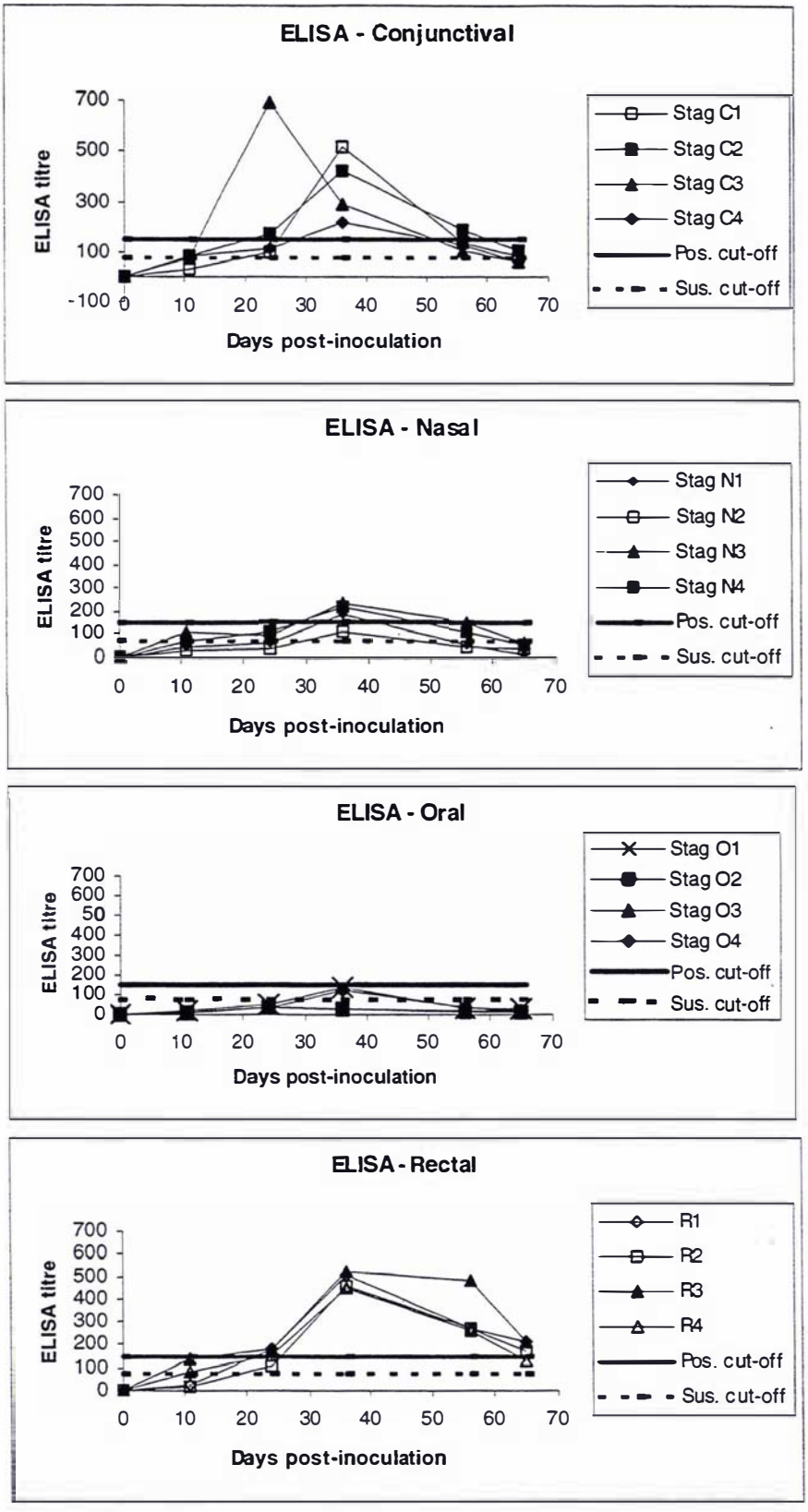


Figure 4.4.2 *B. ovis* ELISA titres for yearling stags artificially infected with *B. ovis* by inoculation of either the conjunctival, nasal, oral or rectal mucous membranes. Stags from which *B. ovis* was isolated from either semen or the reproductive tract at slaughter are denoted by an open symbol.

Table 4.4.1 *B. ovis* culture from the semen, epididymes, seminal vesicles and ampullae of yearling stags artificially inoculated with *B. ovis* by either the conjunctival, nasal, oral or rectal mucous membranes.

Group	Stag ID	Semen day 56	Epididymes day 65	Seminal vesicles day 65	Ampullae day 65
Conjunctival	C1	+	+	-	-
	C2	-	-	-	-
	C3	-	-	-	-
	C4	-	-	-	-
Nasal	N1	NS	-	-	-
	N2	+	+	+	-
	N3	-	-	-	-
	N4	-	-	-	-
Oral	O1	-	-	-	-
	O2	-	-	-	-
	O3	-	-	-	-
	O4	NS	-	-	-
Rectal	R1	+	-	+	-
	R2	+	-	+	+
	R3	NS	-	-	-
	R4	+	-	-	-

\+ *B. ovis* positive
\- *B. ovis* negative
NS: no sample obtained

Table 4.4.2 Histological examination of the epididymes, seminal vesicles and ampullae of stags slaughtered 65 days after artificial infection of *B. ovis* by inoculation of either the conjunctival, nasal, oral or rectal mucous membranes.

Group	Stag ID	Epididymes	Seminal vesicles	Ampullae
Conjunc-tival	C1	+	-	+
	C2	+	-	+
	C3	+	+	+
	C4	-	-	sus
Nasal	N1	-	-	-
	N2	+	-	+
	N3	-	-	-
	N4	sus	-	sus
Oral	O1	-	-	-
	O2	-	-	-
	O3	-	-	-
	O4	sus	sus	-
Rectal	R1	+	-	+
	R2	+	+	+
	R3	+	+	+
	R4	+	+	+

+: lesions consistent with *B. ovis* infection
sus: lesions suggestive of *B. ovis* infection
-: no lesions consistent with, or suggestive of, *B. ovis* infection

4.4.4 Discussion

This experiment demonstrated that stags may become infected and shed *B. ovis* in the semen by contact with the organism onto the conjunctival, nasal or rectal mucous membranes. In this experiment one of four stags became infected by conjunctival inoculation, one of four became infected by nasal inoculation and three of four became infected by rectal inoculation. Similarly, rams have also been experimentally infected by inoculation of these mucous membranes although infection rates have been variable. Following conjunctival inoculation of rams using a *B. ovis* culture, Biberstein et al. (1964) and Paolicchi et al. (2000) demonstrated that 83% and 71% of rams respectively developed infection. However, Brown et al. (1973) found only 7% of rams inoculated in this way became infected. Burgess and Norris (1982) and Plant et al. (1986) infected rams by inoculation of the nasal mucous membranes and found infection in 20% and 33% respectively. In the one published experiment where rams were rectally inoculated,

only one of nine (11%) developed infection (Plant et al., 1986). However, in that trial infected semen was used for inoculation rather than a culture of *B. ovis* organisms.

Brucella ovis was isolated from the semen of a total of five stags, but a further six stags had histological lesions present in the reproductive tract that were consistent with, or suggestive of, *B. ovis* infection. Infections in rams have been described as either "persistent", where localisation of the organism in the epididymes and accessory sex glands occurs, or "transient" where the animal develops serum antibodies but the organism does not localise in the body (Buddle, 1955) and histological lesions are not present in the reproductive tract (Plant et al., 1986). Using these definitions, it would appear that stags C1, N2, R1, and R2 developed persistent infections with localisation of *B. ovis* in the epididymes, seminal vesicles and/or ampullae. It is possible that artificial inoculation of stags C2, C3, C4, N4, O4 and R3 resulted in localisation of *B. ovis* in the epididymes, seminal vesicles and/or ampullae with subsequent cellular changes in these organs, but that these stags then resolved the infection. The persistence of infection in stags is further discussed in Chapter 5. *Brucella ovis* was isolated from the semen of stag R4 collected on Day 56, but not from the reproductive organs of this stag collected at slaughter on Day 65. It is possible that this stag resolved the infection in the intervening nine days or that the organism was still present but was unable to be isolated. It appears that stags N1, N3, O1, O2 and O3 were exposed to the organism (as determined by development of serum antibodies) but overcame the infection before it localised in the reproductive organs and thus were 'transiently' infected.

The presence of serum antibodies gave a good indication of exposure to the organism. Using the CFT, sera from all artificially infected stags with the exception of O3 gave a suspicious or positive reaction on at least one sampling occasion. Using the ELISA, sera from all stags with the exception of O2 and O3 gave a positive or suspicious reaction on at least one sampling occasion. However, by 56 days after inoculation serological titres had decreased in the conjunctival, nasal and oral inoculated stags, even in stags C1 and N2 who were demonstrated to be shedding the organism in semen. In contrast, at 65 days post-inoculation serum antibody titres in the rectal inoculated stags were still in the positive range. This would suggest that serology is good evidence of exposure to infection, provided sampling is done within 30 to 65 days of exposure.

After that, the sensitivity of serology would appear to decrease. The use of serology for diagnosis of *B. ovis* in stags is further discussed in Chapters 5 and 8.

However, these results give cause to question the methodology used to detect infection in stags in Section 4.3, transmission of *B. ovis* between stags by successive grazing or adjacent-paddock grazing. In that experiment serology was used as the method of detecting infection, with subsequent culture of semen and reproductive organs of stags that became serologically positive. Bacteriological culture of semen or reproductive organs from seronegative stags, and histological examination of reproductive organs from all stags was not undertaken. The longest interval between collection of serum samples was 55 days and there is the possibility that within this time period some stags may have become infected but this was not detectable by serology at the next sampling due to decreasing antibody titres. While this is unlikely to have occurred, and the risk of transmission by successive grazing or adjacent-paddock grazing is still considered low, it would have been preferable to have collected blood samples from the non-infected stags at shorter intervals, to have undertaken semen culture of all stags at regular intervals and to have undertaken histological examination of the epididymes, seminal vesicles and ampullae of all stags at slaughter.

Brucella ovis was not isolated from the semen or reproductive organs of any of the four stags inoculated onto the oral mucous membranes, although one stag had mild histological lesions suggestive of *B. ovis* infection. Serological titres from stags in this group were lower than those from the other groups. This suggests that the oral route may be a less important route of infection compared with other mucous membranes. It is likely that this is due to the rapid dilution of organisms with saliva and the robust structure of the buccal mucosa, which has a more protective role than the conjunctival, nasal and rectal mucous membranes. Buddle (1955) and Simmons and Hall (1953) both report infection following oral inoculation in sheep but in neither situation was infection likely to mimic a "natural" infection. Buddle (1955) artificially inoculated nine ewes in early pregnancy with 5ml of an inoculum containing $5x10^9$ colony-forming units per ml administered via an oesophageal tube. Subsequently placental infection was confirmed in five ewes at parturition. Simmons and Hall (1953) inoculated two rams with 80ml and 100ml of an inoculum by oesophageal tube. At 150-160 days after inoculation the

rams were slaughtered and one ram had histological lesions in the reproductive tract consistent with *B. ovis* infection but the organism was not isolated from the reproductive organs of either ram.

In this experiment, serum antibodies were present as early as 11 days after mucosal inoculation. In experiments where rams have been artificially infected by inoculation of mucous membranes it has taken a minimum of 0.5 to two weeks and an average of four weeks for development of detectable serum antibodies (Table 1.3.1); (Laws et al., 1972; Webb et al., 1980; Burgess and Norris, 1982; Plant et al., 1986). This has implications when attempting to eradicate the disease from a flock or herd as it suggests that serological re-testing intervals could be a short as two to three weeks.

A further successful route of infection investigated in experimental studies of rams has been inoculation of the penile or preputial mucosa with *B. ovis* (Table 1.2.1); (Buddle and Boyes, 1953; Jebson et al., 1954; Laws et al., 1972; Webb et al., 1980; Plant et al., 1986; Paolicchi et al., 2000). This route was not investigated in this experiment due to a shortage of available stags, but Chapter 7 details an experiment where stags become infected with *B. ovis* by mating vaginally infected hinds. This suggests that exposure of *B. ovis* to the penile or preputial mucosa of stags also results in infection in this species.

4.5 Observations of the sexual behaviour of groups of stags and rams

4.5.1 Introduction

The mechanism by which transmission of *B. ovis* occurs between rams and between rams and stags is unknown. It has been suggested that transmission of *B. ovis* infection between rams may occur by rectal copulation (Hartley et al., 1955), although the evidence for this is largely circumstantial and is based mainly on the finding of faecal material in the prepuce of rams. A ram has been artificially infected by rectal inoculation (Plant et al., 1986). Ejaculation onto the perineal region and subsequent sniffing or licking of the infected semen by another ram has also been suggested as a route of transmission (Keogh et al., 1958). As documented in Section 4.2, *B. ovis* was

isolated from the prepuce of infected rams and it is possible that it may also be found around the prepuce of infected stags. It is therefore possible that transmission between animals could occur by preputial sniffing or licking. A common behaviour exhibited by stags during the breeding season is to spray fluid from the penis onto the ventral abdomen. This fluid consists of urine and possibly semen (Whitehead, 1993) and a possible route of transmission may be aerosol spray from this fluid contacting the conjunctiva of another animal.

In a naturally-occurring case of *B. ovis* infection in rising-three-year old stags in the Canterbury region of New Zealand, 88% of a group of 34 stags had positive or suspicious serological titres to *B. ovis*, indicating a very high infection rate (Scott, 1999). This would suggest that transmission of infection between stags could be due to a common behavioural event.

The objective of this study was to identify whether stags and rams in same-sex groups display behaviour that could aid the transmission of *B. ovis*.

4.5.2 Materials and methods

Study periods, locations, animals and observation hours

Three studies were carried out to investigate sexual behaviour of groups of rams or stags. Details of the numbers of animals, their ages and the number of observational hours are included in Table 4.5.1.

Study A - observations of the behaviour of rams and stags were carried out at the Massey University Deer Research Unit and the Massey University Veterinary Large Animal Teaching Unit. Two groups of rams were observed for a total of 35 hours from February 12 to April 15, 1999. Four groups of stags were observed for a total of 44 hours from March 17 to May 10 1999.

Study B Eight groups of stags from five commercial deer properties in the Manawatu district were observed for a total of 36 hours from April 13 to April 29, 1999.

Study C Three groups of stags, one of the Massey University Deer Research Unit and two on commercial deer properties located in the Manawatu district, were observed for a total of 60 hours from March 14 to May 12, 2000.

In the three studies, rams were observed for a total of 35 hours and stags were observed for a total of 140 hours. In all studies, observations occurred at varying times of the day. In Study B, one 2.5-hour observation period was undertaken at night using an infra-red video camera. The studies occurred during the natural breeding period for sheep and deer in New Zealand.

Observers

Observations were undertaken by Agricultural students from Wageningen University in the Netherlands who came to New Zealand for three to four month placements. Bart Tas undertook Study A, Paul Stoutjesdijk and Harro Timmerman undertook Study B while Lars Gorisse and Willem de Klein undertook Study C.

Observation method

Rams were individually identified by spray-painting a number on the back, and observed either in a paddock ranging in size from one to two hectares or in a yard 32m^2 in size. In the paddock, observations were carried out by an observer approximately 50 metres away on a hill using binoculars. In the yard study, rams were observed from a 1.5m high fence at a distance of 5m from the yard.

In Study A, stags were observed by a person stationed in a caravan sited in an adjacent paddock. The caravan had been placed there 10 days prior to the observation study commencing to allow the stags to habituate to it. The stags were in paddocks one hectare in size. Each stag was identified with a different coloured neck collar.

In Studies B and C, stags were observed from a car driven onto the property and sited next to the paddock so there was no opportunity for stags to become habituated to the observational vehicle between observations. Paddock sizes ranged from 1.5 to four hectares in size. Stags were not individually identified.

Observers used a tabular recording sheet on which the behaviours were listed horizontally and five-minute periods were marked vertically. The frequency of each behaviour was recorded during each five-minute period. The date, start and end-time, number and age of stags or rams in the group, paddock area and weather conditions were also recorded.

Recorded behaviours

The following behaviours, considered high-risk for transmission of *B. ovis*, were recorded:

- mounting. Stags may display "low-mounting" behaviour where the sternum of the mounting stag rests on the rump of the deer being mounted (Figure 4.5.1), (Veltman, 1985). Intromission does not occur. They may also display "high-mounting" behaviour where the stag stands almost vertically on the hind feet behind the deer being mounted (Veltman, 1985). Intromission and ejaculation occur only during the high mount. However, it was not possible to observe whether this event actually occurred during visual observations because high mounts are very rapid. Both low-mounts and high-mounts were recorded
- preputial sniffing/licking of the preputial region of another animal (Figure 4.5.2)
- sniffing/licking of the perineal region of another animal
- spraying fluid from an extruded penis

The frequency of each behaviour per animal per hour was calculated by dividing the number of times the behaviour was observed by the number of animals, and then dividing this by the number of observational hours (f = no. observations/ no. animals/ no. hours).

Figure 4.5.1 "Low mounting" behaviour between stags, where the mounting stag rests his sternum on the rump of the stag being mounted.

Figure 4.5.2 A stag sniffing or licking the preputial region of another stag.

4.5.3 Results

In the ram groups of Study A, a total of 145 mounts, 76 instances of preputial sniffing and 334 instances of perineal sniffing were observed with frequencies of 0.09, 0.05 and 0.2 per ram per hour respectively (Table 4.5.1). In two of the four stag groups a total of 11 mounts were observed at a frequency of 0.15 and 0.18 mounts per stag per hour respectively (Table 4.5.1). All were low mounts. Two instances of preputial sniffing were observed in one group of stags, equating to a frequency of 0.06 per stag per hour. Perineal sniffing was observed in three of the four groups with frequencies of 0.001 to 0.006 per stag per hour (Table 4.5.1).

During Study B, a total of 22 mounts, all of which were low mounts, were observed in three of the eight groups of stags equating to a frequency of 0.03, 0.04 and 0.01 mounts per stag per hour respectively (Table 4.5.1). Preputial sniffing was observed in only one group of stags, at a frequency of 0.001 sniffs per hour. Perineal sniffing was observed in two groups of stags at a frequency of 0.03 and 0.005 per stag per hour respectively and stags spraying fluid from an extruded penis was observed in five of the eight groups at a frequency of 0.01 to 0.05 per stag per hour (Table 4.5.1).

During Study C, none of the recorded behaviours were observed in one of the three groups of stags whereas the remaining two groups displayed all four behaviours. A total of 10 high mounts and 164 low mounts were observed giving frequencies of 0.09 and 0.03 mounts per stag per hour. Preputial sniffing was observed on 26 occasions, equating to 0.017 and 0.004 per stag per hour. Perineal sniffing was observed 97 times equating to 0.06 and 0.02 per stag per hour and stags spraying fluid from an extruded penis were observed on 48 occasions, at a frequency of 0.02 and 0.01 per stag per hour (Table 4.5.1).

It was noticed that during the low mount the mounting stag extruded the penis but it was not possible to determine if it ejaculated onto the perineum of the stag being mounted.
It was also noted that other animals did not appear to show an increased interest in sniffing or licking the perineal region of an animal that had just been mounted or an animal that had just undertaken mounting behaviour.

Table 4.5.1 Number and frequency (n and f) of high and low mounting behaviour, preputial sniffing, anal sniffing and spraying fluid from an extruded penis observed in groups of rams and stags during the breeding periods of 1999 (Studies A and B) and 2000 (Study C).

				Mounts		Preputial sniffing		Perineal sniffing		Spraying , extr. penis	
Study and species	**Age of animals**	**No. of animals**	**Obs. hours**	**n**	**f**	**n**	**f**	**n**	**f**	**n**	**f**
A: Rams	R2	20	17	10	0.029	10	0.029	29	0.085	n/r	-
	MA	25	18	135	0.300	66	0.147	305	0.678	n/r	-
A: Rams; Total		**45**	**35**	**145**	**0.091**	**76**	**0.048**	**334**	**0.212**	**-**	**-**
A: Stags	R2	6	11	0	0	0	0	1	0.015	n/r	-
	R2	6	11	0	0	0	0	0	0	n/r	-
	R2	3	11	5	0.151	0	0	2	0.061	n/r	-
	R2	3	11	6	0.182	2	0.061	2	0.061	n/r	-
A: Stags; Total		**18**	**44**	**11**	**0.014**	**2**	**0.003**	**5**	**0.006**	**-**	**-**
B: Stags	R2	60	3	0	0	0	0	0	0	0	0
	R2	20	3	0	0	0	0	0	0	0	0
	R3	21	3	2	0.032	0	0	2	0.032	0	0
	R5	17	4.5	3	0.039	0	0	0	0	2	0.026
	R5	14	1	0	0	0	0	0	0	2	0.014
	R7	11	5.5	0	0	0	0	0	0	3	0.050
	MA	18	5	0	0	0	0	0	0	4	0.044
	MA	120	11	17	0.013	9	0.007	7	0.005	14	0.011
B: Total		**281**	**36**	**22**	**0.002**	**9**	**0.001**	**9**	**0.001**	**25**	**0.002**
C: Stags	R3	17	3	0	0	0	0	0	0	0	0
	MA	25	35	71 (10)	0.081 (0.011)	15	0.017	51	0.058	16	0.018
	MA	133	22	93	0.032	11	0.004	46	0.016	32	0.011
C: Total		**199**	**60**	**174**	**0.015**	**26**	**0.002**	**97**	**0.008**	**48**	**0.004**

R2, R5 etc. - rising-2-years of age, rising-5-years of age etc.
MA - mixed age
n/r – not recorded
( ) = high mounts, all others were low mounts

4.5.4 Discussion

The objective of this study was to determine, in an epidemiological rather than an ethological context, whether behaviours likely to be of risk for transmission of *B. ovis* occur in groups of rams or stags, and to broadly quantify their frequency. During these studies it was observed that rams and stags both exhibited mounting, preputial sniffing and perineal sniffing behaviours that are likely to be high risk for the transmission of *B. ovis*. In addition, stags were also observed to spray fluid from an extruded penis which is also likely to be high risk for transmission.

Mounting behaviour and preputial and perineal sniffing were relatively common behaviours amongst the rams, particularly the mixed-age group of rams. The frequency of mounting behaviour in the mixed-age rams was 0.3 mounts per ram per hour, similar to the 0.14 mounts per ram per hour observed by Zenchak et al. (1981) but less than the 1.08 and 1.43 mounts per ram per hour reported by Price et al. (1988) and Katz et al. (1988) respectively. In both the current study and published studies it was not determined whether rectal copulation occurred during mounting although it is likely to have occurred. This behaviour should provide opportunity for transmission of *B. ovis*. Preputial and perineal sniffing were also relatively frequent behaviours observed in the rams, particularly the mixed-age rams. Thwaites (1982) also observed rams sniffing or licking the perineal region and prepuce of one another, although the frequency of these behaviours was not reported. *Brucella ovis* has been isolated from the prepuce of rams (Section 4.2) and therefore preputial sniffing may result in *B. ovis* contacting the nasal mucous membrane resulting in infection. Similarly, sniffing semen deposited around the perineal region of a ram after it has been mounted and ejaculated on may also result in transmission.

In this study the mixed-age rams showed 5-10 times the frequency of sexual behaviours as the rising-two-year old rams, although other researchers have found a high frequency of sexual activity in young rams (Price et al., 1988; Katz et al., 1988).

There was a wide variation in the frequency of behaviours between the stag groups, with some behaviours not observed in some groups whereas other groups were relatively

active. These differences did not appear to be related to the age of the stags or the size of the group. It was noticed by the observers that sexual activity tended to occur in sporadic "bursts", where there would be a flurry of activity within a five to 10 minute period and then no activity for the remaining hours. This may explain the apparent differences in the frequency of these behaviours between the groups. Other factors that may have been related to these differences are the time of day during which observations were undertaken, the weather patterns at the time, the proximity of the nearest group of hinds and the size of the paddock.

A total of 10 high mounts were observed during these studies. All high mounts were observed in the same group of stags but it is likely that this behaviour occurs at a very low frequency in most groups of stags. It is unknown whether rectal copulation occurred during the high mounts, but experimental inoculation of the rectal mucous membranes (Section 4.4) resulted in three of four stags becoming infected, suggesting an ease of infection by this route. Therefore if high-mounting and rectal copulation of an infected onto a non-infected stag did occur then the risk of transmission of infection is likely to be high.

In the stag groups 187 of the 197 mounts observed, or 95%, were low mounts. Similarly, Veltman (1985), while observing the mating behaviour of stags joined with groups of hinds over a three-year period, reported that 75 to 87% of stag-on-hind mounts were low mounts. Because copulation does not occur during low mounting (Veltman, 1985) then in itself this behaviour is unlikely to result in transmission of *B. ovis*. However, if the mounting stag ejaculated onto the perineum of the animal being mounted, this would provide a source of infective semen. It has been demonstrated that stags can become infected with *B. ovis* by inoculation of the nasal mucosa (Section 4.4), suggesting a possible route of transmission. Perineal sniffing behaviour was observed in seven of the 15 groups of stags while preputial sniffing was observed in four of the 15 groups of stags. Likewise, spraying fluid from an extruded penis was observed in seven of 11 groups of stags and it is possible that contact of this fluid with conjunctival or nasal mucous membranes may result in infection. Experimentally, stags have become infected by inoculation of these mucous membranes (Section 4.4).

There is little published data available on the sexual behaviour of stags in all-male groups but in a study of the behaviour of Elk in a wild environment in California over a two-year period, Harper et al. (1967) observed only one instance of mounting behaviour between stags when an adult stag mounted a young stag. In the wild environment, stags remain in all-male groups outside the rut period but during the rut these groups fragment and individual stags move to a rutting ground where they join with harems of hinds (Clutton-Brock and Albon, 1989). It is probable that domestication and farming of stags in all-male groups has resulted in a different expression of sexual behaviour between stags compared with the wild environment, thus creating the circumstances that permit rapid transmission of *B. ovis* in stag groups. Mounting behaviour amongst males of other species can be an expression of dominance (Dagg, 1984) and this may also be the motivation for mounting behaviour between stags. Thus it is possible that if a dominant stag rather than a stag low in the social hierarchy initially became infected, the disease would spread more rapidly.

It has been demonstrated that stags can become infected with *B. ovis* from contact with infected rams (Section 4.2) but the mechanism of transmission between the species is unknown. It is possible that transmission occurs by stags sniffing the prepuce of rams or sniffing semen ejaculated onto the perineum of rams after being mounted by another ram. However, observations of rams and stags together have not been undertaken and further research would be required to determine what interaction the two species show towards one another.

4.6 General discussion

These experiments, and the work of Barron (1984) and West et al. (1999) have demonstrated transmission of *B. ovis* from stag to stag and from ram to stag. Transmission is likely to occur only when animals are in direct contact with one another. The route of infection between stags and from rams to stags is still unknown but stags have become infected with *B. ovis* by inoculation of the nasal, conjunctival, penile and rectal mucosa suggesting that infection may result from infected semen contacting any of these mucous membranes.

There are potentially four ways by which stags may become infected:

Preputial sniffing or licking

Brucella ovis was isolated from the prepuce of four of five infected rams that were shedding the organism in semen (Section 4.2) and it is possible that it may also reside around the prepuce of stags. It has been demonstrated that stags can become infected with *B. ovis* by inoculation of the nasal mucous membranes and it is therefore possible that stags sniffing the prepuce of infected rams or other stags may be an important route of transmission. Behavioural observations of groups of stags have shown that they do engage in preputial sniffing and licking.

Anal sniffing/licking after mounting behaviour with ejaculation onto this region

If an infected ram mounts another ram or an infected stag mounts another stag and subsequently ejaculates onto the perineal region of the mounted animal, then this depot of semen could potentially be infective to either a third animal that sniffs or less likely licks it. Keogh et al. (1958) suggested that this may be an important route of transmission between rams.

Aerosol spray of infective semen or urine

Behavioural observations have identified that stags spray fluid from an extruded penis during the breeding season, and therefore aerosol spray of organisms contacting the conjunctivae or nostrils may also be an important route of transmission. It has not been ascertained whether the sprayed fluid contains semen, but even if it were only urine it is possible that residual semen from the urethra may be mixed with it. Biberstein et al. (1964) demonstrated that *B. ovis* can be isolated from the kidneys of infected rams and the organism has been isolated from the urine of infected rams (Chapter 5).

Rectal copulation

It has been suggested that rectal copulation may be an important means of transmission of *B. ovis* between rams (Jebson et al., 1954; Hartley et al., 1955) although this concept was based on the observation of rams frequently mounting one another and the finding of faecal matter in the prepuce. It has not been quantified or proven. Inoculation of the rectal mucous membranes of stags was an effective way of inducing infection although

the behavioural studies outlined in Section 4.5 would suggest that high mounting and possible rectal copulation are uncommon activities in groups of stags, whereas other behaviours such as preputial or anal sniffing and fluid spraying are more common. While rectal copulation as a route of transmission for *B. ovis* between stags cannot be discounted, it may be a less likely route than others. Transmission of infection from rams to stags could not have occurred by this route.

4.7 Conclusions

1) Transmission of *B. ovis* can occur from infected rams to stags grazing in the same paddock.

2) Grazing disease free stags in a paddock just vacated by *B. ovis* infected stags is unlikely to result in transmission of infection.

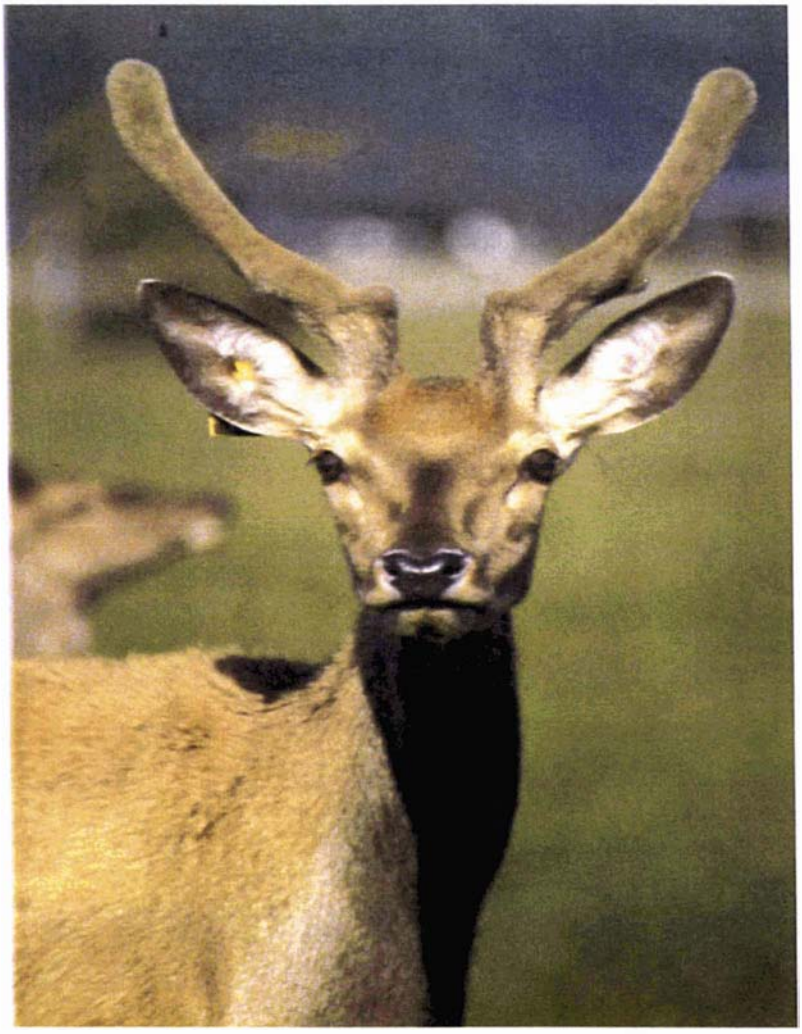
3) Grazing disease free stags in a paddock adjacent to *B. ovis* infected stags is unlikely to result in transmission of infection between the two groups.

4) Groups of stags and groups of rams demonstrate sexual behaviours that are considered high-risk for transmission of *B. ovis*

5) Stags were successfully infected with *B. ovis* by inoculation of the conjunctival, nasal and rectal mucous membranes.

Chapter Five

A longitudinal study of *Brucella ovis* infection in stags and rams



5.1 Introduction

In a previous experiment it was observed that *B. ovis* infection in stags appeared to resolve earlier than would be expected in rams. Five stags artificially infected by intravenous inoculation developed patent infections and *B. ovis* was isolated from their semen seven months (217 days) after inoculation (Section 4.3). However, when these stag were slaughtered 12 months after inoculation, *B. ovis* was not cultured from the reproductive organs, suggesting that the infection had resolved. In addition, it was observed that the serological titres of the infected stags tended to decline earlier than would be expected in rams, which also raised the possibility that serology may fail to detect stags that have been infected for some months.

While there is extensive data available on the establishment of *B. ovis* infection in rams (Table 1.2.1), as well as the sensitivity and specificity of serological tests (Section 1.5) and the pathology of infection (Section 1.3), there have been few published experiments investigating infection in a group of rams over a period of time.

The objective of this experiment was to undertake a longitudinal study to determine the establishment and persistence of artificially induced *B. ovis* infection and to investigate the serological responses, and the pathology of the reproductive tract in long-term infections of stags and rams.

5.2 Materials and methods

General sampling and analysis methods are detailed in Chapter 2. A timeline of the series of events in this experiment is summarised in Figure 5.1.

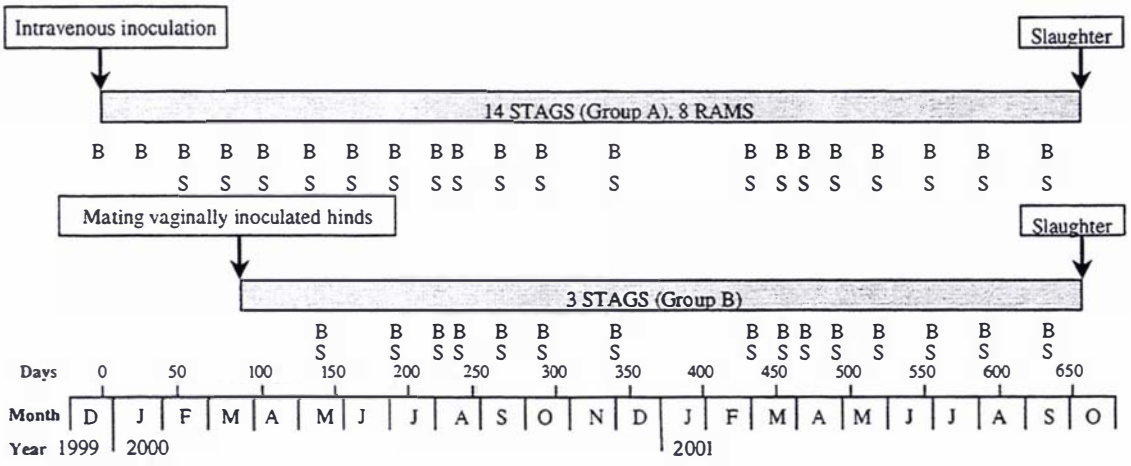


Figure 5.1 Timeline of events for an experiment investigating the establishment, persistence and serological responses of *B. ovis* infection in stags and rams. B = blood samples collected, S = semen samples collected.

5.2.1 Animals and infection

Fourteen initially seronegative 12-month-old red deer stags (Group A) and eight 16-month-old Romney rams were artificially infected with *B. ovis* by injection of 1ml of an inoculum containing $1x10^9$ colony forming units into the jugular vein on December 23, 1999. The animals were slaughtered 649 days later on October 3, 2001.

During March 2000 the stag group was increased to 17 by the addition of three 15-month-old red deer stags (Group B). These stags had become infected by mating hinds synchronised for oestrus and that had received approximately $1x10^8$ colony forming units of *B. ovis* into the vagina immediately before their first and second oestrus periods (Chapter 7). These three stags were slaughtered on October 3 2001, approximately 561 days after becoming infected.

Throughout the experiment, the stag group was kept on the Massey University Deer Research Unit while the ram group was kept separately on the Massey University Large Animal Teaching Unit (Section 2.2).

5.2.2 Sampling of animals

Blood and semen samples were collected from stags and rams at approximately monthly intervals (Figure 5.1). Blood samples only were collected from Group A stags and rams on days 18 and 55 after inoculation. Blood and semen samples were collected from Group A stags and rams on Days 83, 103, 138, 166, 200, 223, 235, 263, 291, 342, 437, 459, 467, 493, 524, 558, 592 and 630 after inoculation. By using rectal ultrasonography of the hinds to estimate foetal age and therefore the approximate mating date (Chapter 7) it was estimated that infection was established in the three Group B stags on approximately March 22, 2000. Blood and semen samples were collected from the Group B stags on Days 57, 77, 134, 146, 174, 202, 253, 349, 371, 379, 405, 435, 470, 504 and 542 after infection.

Group A stags and rams were slaughtered 649 days after intravenous inoculation, and Group B stags were slaughtered 561 days after exposure to infection.

5.2.3 Serology

Serum samples were collected and tested in a *B. ovis* CFT and ELISA performed at a commercial laboratory as detailed in Section 2.5. The sensitivity of these tests was calculated by dividing the number of animals identified as positive or suspicious in the test by the number of animals that were infected (as defined by the isolation of *B. ovis* from semen), and multiplying the result by 100 to give a percentage value. The cut-off levels of the tests were those described for sheep (Section 2.5).

5.2.4 Pathology and microbiology

Semen samples were collected for *B. ovis* culture on the days specified in Section 5.2.2 (Figure 5.1). On Days 83, 103, 263, 437 and 524 the scrotal contents of the stags and rams were palpated and lesions of epididymitis were recorded. At slaughter, the epididymes, seminal vesicles and ampullae were collected for gross pathological and histopathological examination, and *B. ovis* culture.

For comparative purposes, the epididymes, seminal vesicles and ampullae were examined from eight stags from the same property. These eight stags had never been in contact with *B. ovis* infected animals and on the basis of serology and culture of the reproductive tract they were found to be non- infected with *B. ovis*.

5.3 Results

5.3.1 Culture

Group A stags

Brucella ovis was cultured from the semen of 12 of the 14 Group A stags on Days 83 and 103, confirming that a patent infection had been established (Table 5.1). However, by Day 342 after inoculation *B. ovis* was no longer isolated from the semen of 10 of these 12 stags, nor was it isolated from the epididymes, seminal vesicles or ampullae of these stags at slaughter on Day 649. The organism was isolated from the semen of two stags, numbers 803 and 827, throughout the experiment (Table 5.1) and from the reproductive organs at slaughter on day 649 (Table 5.4). *Brucella ovis* was not isolated from the semen of stags 826 or 861 at any of the sampling times.

Table 5.1 *B. ovis* culture from the semen of 14 stags (Group A) artificially infected with *B. ovis* by intravenous inoculation on Day 0.

Stag ID	DAYS AFTER INOCULATION																
	83	103	138	166	200	223	235	263	291	342	437	459	467	493	524	558	592	630
803																		
827				NS										NS				
842																		
852			NS												NS		NS	
854														NS				
855			NS										NS					
860													NS			NS		
864																		
866																		
869							NS			NS			NS					
865	NS			NS	NS		NS									NS		
867																NS		
826																NS		
861			NS															

B. ovis positive

B. ovis negative

NS no semen sample collected

Group B stags

Brucella ovis was cultured from the semen of all three Group B stags on Days 57 and 77 (Table 5.2). However, on Day 134 and at all subsequent sampling times, *B. ovis* was not isolated from the semen from stag 833. On Day 253 and all subsequent sampling times the organism was not isolated from the semen from stags 839 and 850. *Brucella ovis* was not isolated from the epididymes, seminal vesicles or ampullae of any of the three stags at slaughter approximately 561 days after infection.

Table 5.2 *B. ovis* culture from the semen of three stags infected with *B. ovis* by mating vaginally infected hinds on approximately Day 0.

Stag	DAYS AFTER EXPOSURE TO INFECTION														
ID	55	77	134	146	174	202	253	349	371	379	405	435	470	504	542
833	■	■												NS	
839	■	■	■	■	■	■									
850	■	■	■	■	■	■			NS			NS			

■ *B. ovis* positive

B. ovis negative

NS no semen sample collected

Rams

Brucella ovis was cultured from the semen of six of the eight rams, confirming that a patent infection had been established (Table 5.3). The organism was isolated from the semen of five of the eight rams at all sampling times and from the semen of one ram, number 61, at 15 of the 18 sampling times (Table 5.3). At slaughter on Day 649 the organism was isolated from the epididymes of all six of these rams, the seminal vesicles of five, the ampullae of four and the urinary bladder of four (Table 5.4). *Brucella ovis* was not cultured from the semen of two rams, numbers 55 and 56, at any of the sampling times (Table 5.3) and it was not isolated from the reproductive organs of these rams at slaughter on Day 649.

Table 5.3 *B. ovis* culture from the semen of eight rams artificially infected with *B. ovis* by intravenous inoculation on Day 0.

Ram ID	DAYS AFTER INOCULATION																	
	83	103	138	166	200	223	235	263	291	342	437	459	467	493	524	558	592	630
55																		
56																		
57																		
58																		
59																		
60								NS										
61																		
69																		

B. ovis positive

B. ovis negative

NS no semen sample collected

Table 5.4 *B. ovis* culture from the epididymes, seminal vesicles, ampullae and urinary bladder of two stags and six rams infected with *B. ovis* by intravenous inoculation on Day 0 that shed *B. ovis* in semen for the following 630 days. Animals were slaughtered after 649 days.

Species/ID	Epididymes	Seminal vesicles	Ampullae	Urinary bladder
Cervine/803	+	+	-	-
Cervine/827	+	NS	NS	NS
Ovine/57	+	+	+	+
Ovine/58	+	+	+	+
Ovine/59	+	+	-	+
Ovine/60	+	+	+	-
Ovine/61	+	-	-	-
Ovine/69	+	+	+	+

+ *B. ovis* positive

- *B. ovis* negative

NS: no sample collected due to a communication error

5.3.2 Serology

For ease of presentation of individual animal serological results, the 17 stags and eight rams involved in this experiment have been grouped into:

- two stags and six rams from which *B. ovis* was isolated from semen throughout the 630 day sampling period
- four stags that stopped shedding *B. ovis* in semen 103 to 263 days after inoculation
- six stags that that stopped shedding *B. ovis* in semen 263 to 342 days after inoculation
- three stags that became infected by mating vaginally infected hinds
- two stags and two rams from which *B. ovis* was not isolated from semen at any time during the 630 day sampling period

Raw serological data is presented in Appendix 1 (Tables A1.6, A1.7, A1.8, A1.9, A1.10, A1.11).

Stags and rams shedding B. ovis in semen throughout the sampling period

Sera from the two stags, in this category (numbers 803 and 827) were positive or suspicious in the CFT and ELISA until Day 223. Between Days 223 and 437, sera from these stags were suspicious or negative in both the CFT and ELISA. From Days 459 to 630 sera were positive or suspicious in the CFT and from Day 437 to 630, sera were positive or suspicious in the ELISA (Figures 5.2, 5.4). Sera from the six rams in this category were positive or suspicious in both the CFT and ELISA throughout the 630-day sampling period, with the exception of ram 59 whose serum was negative in the CFT on Day 630 (Figures 5.3, 5.5).

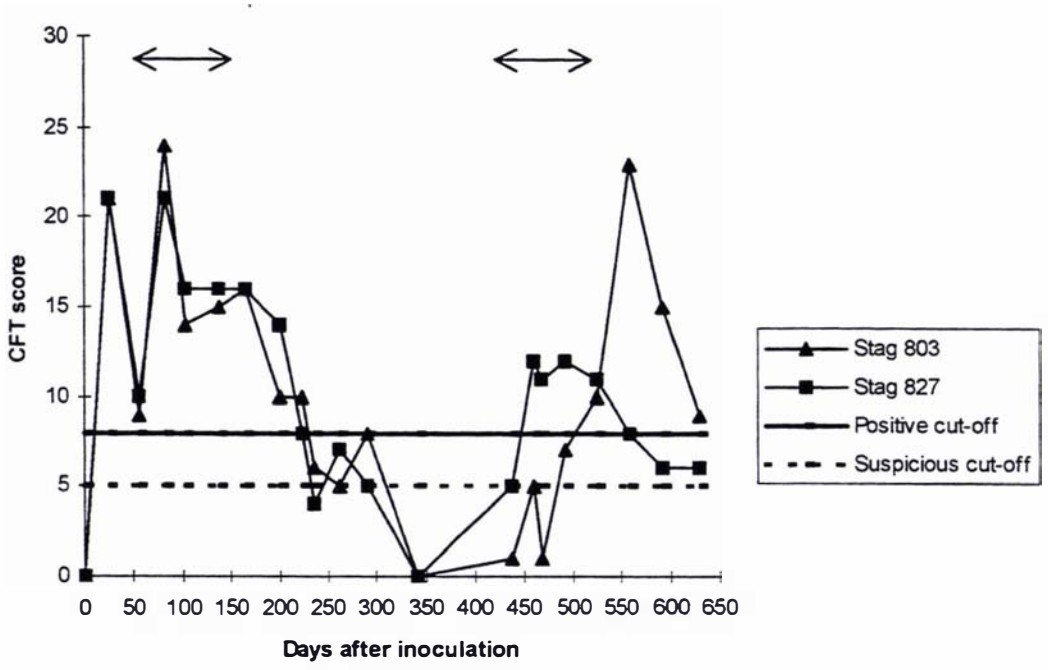


Figure 5.2 *B. ovis* CFT scores from sera collected from two stags intravenously inoculated on Day 0 that shed *B. ovis* in semen throughout the 630-day sampling period. Arrows indicate the breeding periods.

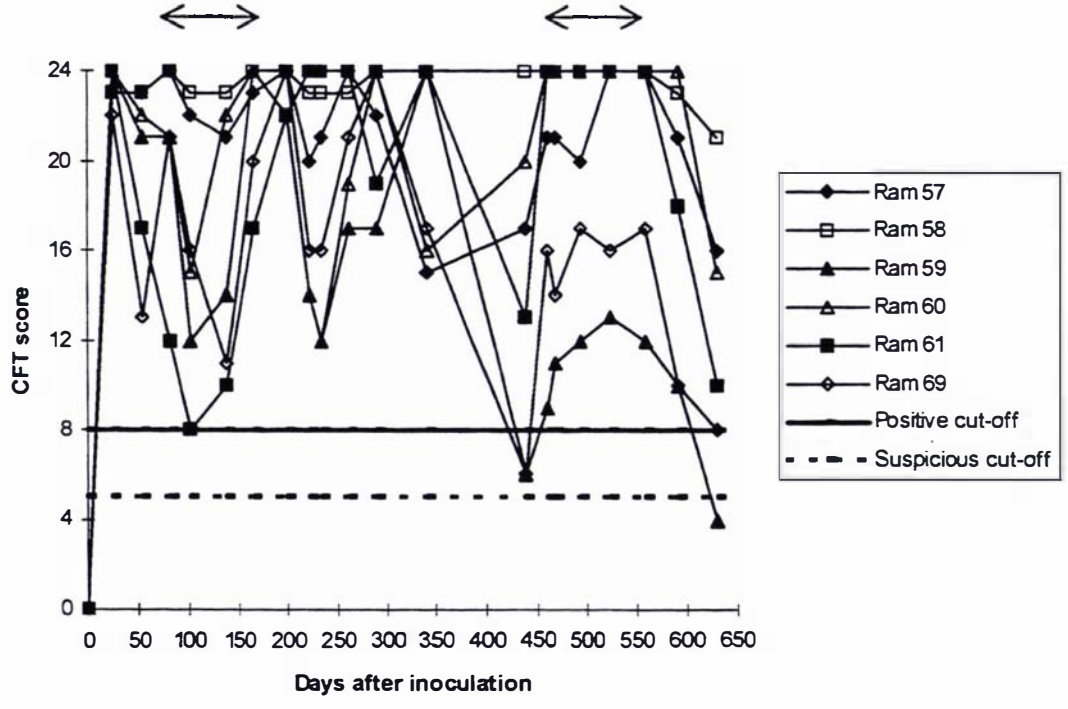


Figure 5.3 *B. ovis* CFT scores from sera collected from six rams intravenously inoculated on Day 0 that shed *B. ovis* in semen throughout the 630-day sampling period. Arrows indicate the breeding period.

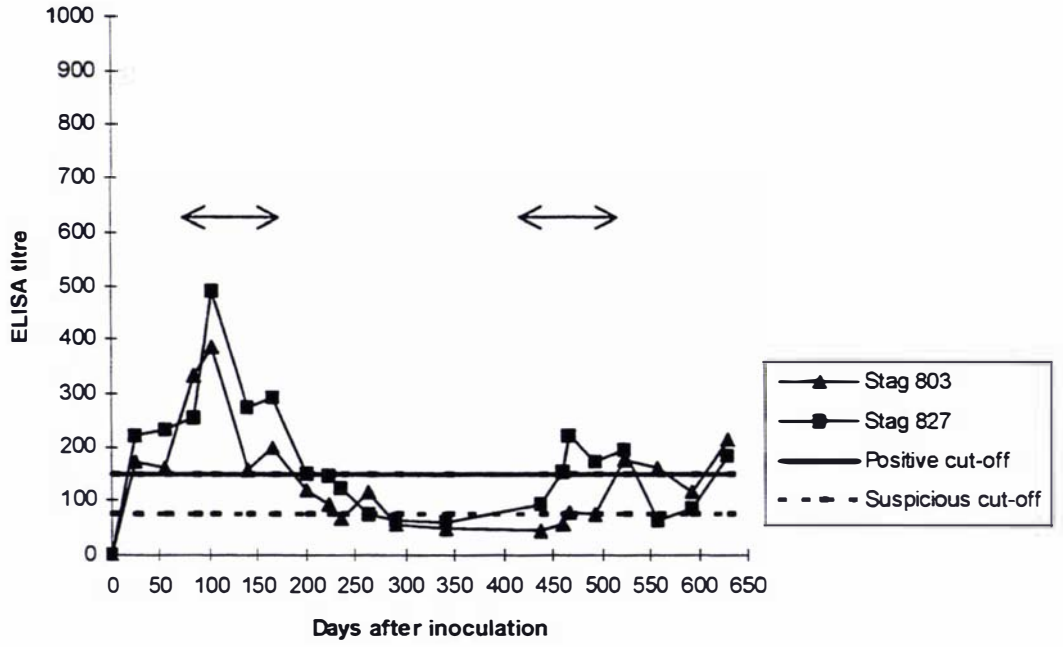


Figure 5.4 *B. ovis* ELISA titres from sera collected from two stags intravenously inoculated on Day 0 that shed *B. ovis* in semen throughout the 630-day sampling period. Arrows indicate the breeding period

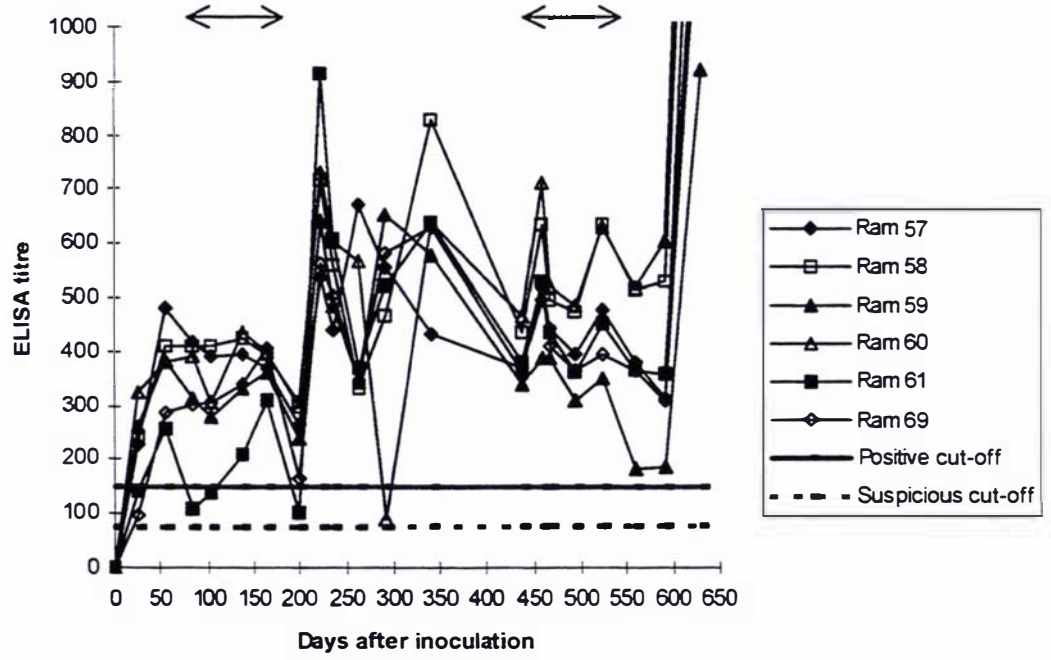


Figure 5.5 *B. ovis* ELISA titres from sera collected from six rams intravenously inoculated on Day 0 that shed *B. ovis* in semen throughout the 630-day sampling period. Arrows indicate the breeding period.

Stags that stopped shedding B. ovis in semen between 103 and 263 days after inoculation

Four stags intravenously inoculated with *B. ovis* on Day 0 shed *B. ovis* in semen for a period of 103 - 235 days after infection (Table 5.1; stags 842, 854, 865 and 867). After this time, *B. ovis* was no longer isolated from the semen of these stags, nor was it isolated from the reproductive organs of these stags at slaughter on Day 649.

On Day 138, *B. ovis* was no longer isolated from the semen of stag 867. Sera from this stag were positive in the CFT and ELISA until Day 138 and then negative in both tests until the end of the experiment (Figures 5.6, 5.7).

On Day 200, *B. ovis* was no longer isolated from the semen of stag 854. Sera from this stag were positive in the CFT and positive or suspicious in the ELISA until Day 291. Sera were negative in both tests until Day 437 and then positive or suspicious in the CFT until Day 592. On Day 630, serum from this stag was negative in the CFT but suspicious in the ELISA (Figures 5.6, 5.7).

On Day 223, *B. ovis* was no longer isolated from the semen of stag 865. No semen samples were collected from the previous two attempts on Days 16 and 200. Sera from this stag were positive in the CFT and ELISA until Day 166 and then negative in both tests until the end of the experiment (Figures 5.6, 5.7).

On Day 263, *B. ovis* was no longer isolated from the semen of stag 842. Sera from this stag were positive or suspicious in the CFT and ELISA up until Day 200 and then negative in both tests until Day 493. From Day 493 until Day 630, sera from this stag were often positive or suspicious in the CFT or ELISA (Figures 5.6, 5.7).

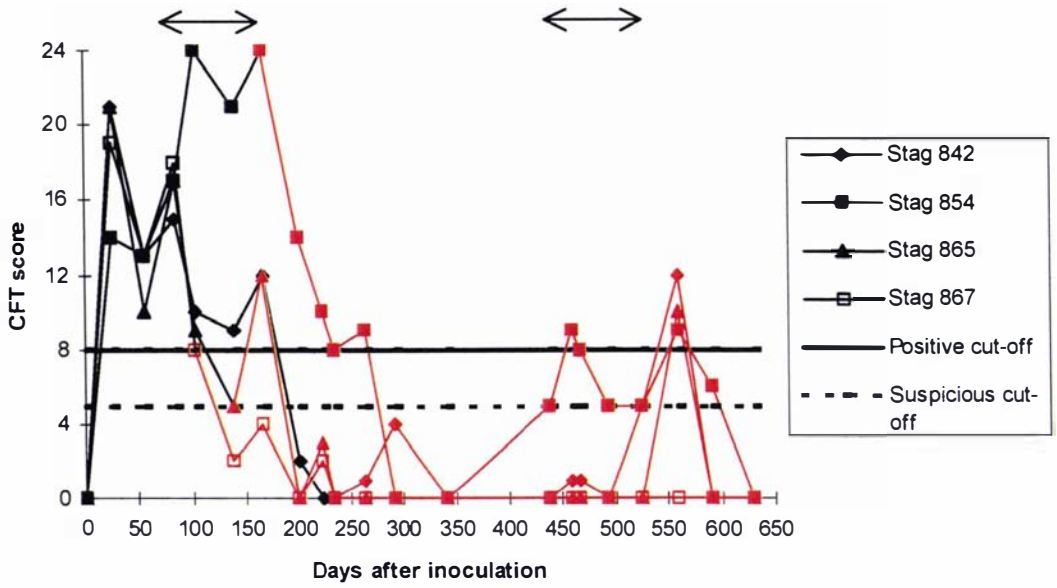


Figure 5.6 *B. ovis* CFT scores for four stags intravenously inoculated on Day 0 that stopped shedding the organism in semen between 103 and 263 days after inoculation. A black symbol denotes that *B. ovis* was isolated from the semen of the stag while a red symbol denotes that *B. ovis* was not isolated. Arrows indicate the breeding periods.

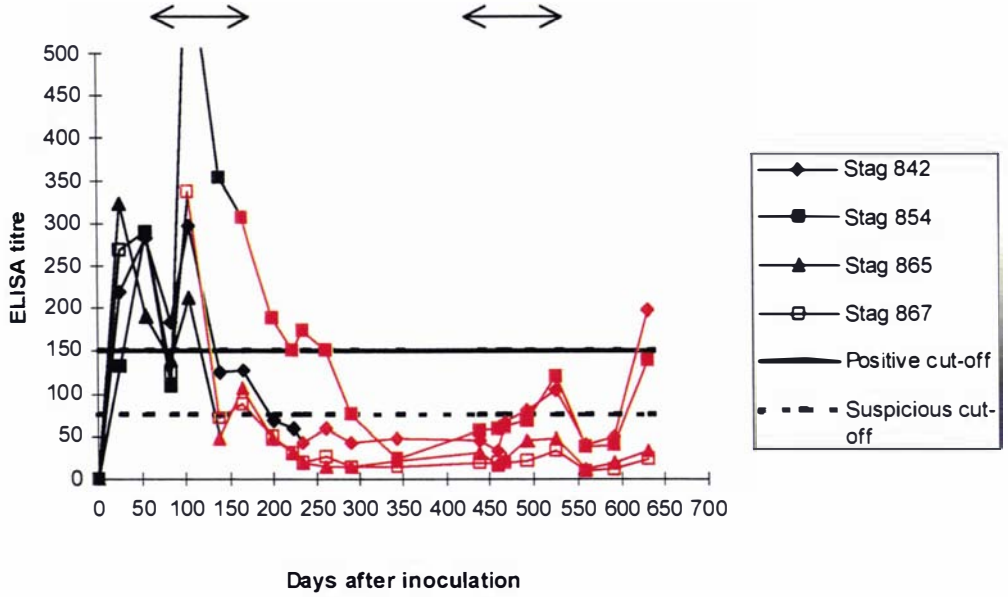


Figure 5.7 *B. ovis* ELISA titres for four stags intravenously inoculated on Day 0 that stopped shedding the organism in semen between 103 and 263 days after inoculation. A black symbol denotes that *B. ovis* was isolated from the semen of the stag while a red symbol denotes that *B. ovis* was not isolated. Arrows indicate the breeding periods.

Stags that stopped shedding B. ovis in semen between 263 and 342 days after inoculation

Six stags intravenously inoculated with *B. ovis* on Day 0 shed *B. ovis* in semen for a period of 263-291 days after infection (Table 5.1.1; stags 852, 855, 860, 864, 866 and 869). After this time, *B. ovis* was no longer isolated from the semen of these stags, nor was it isolated from the reproductive organs of these stags at slaughter on Day 649.

On Day 291, *B. ovis* was no longer isolated from the semen of stags 864 and 869. Sera from these stags were positive or suspicious in the CFT until Day 342 and in the ELISA until Day 235. Sera from both stags were then negative in the ELISA until slaughter. Serum from stag 869 gave a suspicious reaction in the CFT on Days 459 and 524 but other sera from both stags were negative in the CFT from Day 342 until Day 630 (Figures 5.8, 5.9).

On Day 342, *B. ovis* was no longer isolated from the semen of stags 852, 855, 860 and 866. Sera from stag 852 were positive or suspicious in the CFT and ELISA until Day 166, negative in the CFT and suspicious on the ELISA on Day 200 and then negative in the CFT for the remainder of the experiment but positive or suspicious in the ELISA from Days 493 to 630. Sera from stag 855 were positive or suspicious in both tests until Day 200, then negative in both tests until Day 630. Sera from stag 860 were positive or suspicious in both tests until Day 166, suspicious in the CFT but negative in the ELISA on Days 200 and 263, and negative in both tests until Day 630. Sera from stag 866 were positive or suspicious in both tests until Day 291 and negative in the CFT but suspicious in the ELISA on Day 342. From Day 437, sera from this stag were intermittently positive or suspicious in both the CFT and ELISA (Figures 5.8, 5.9).

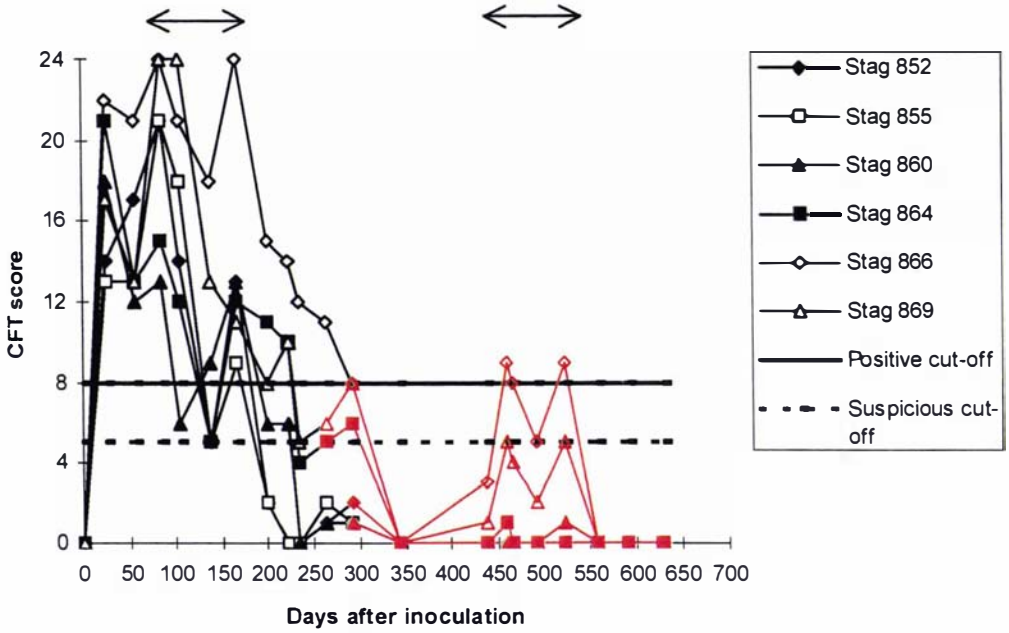


Figure 5.8 *B. ovis* CFT scores for six stags intravenously inoculated on Day 0 that stopped shedding the organism in semen between 263 and 342 days after inoculation. A black symbol denotes that *B. ovis* was isolated from the semen of the stag while a red symbol denotes that *B. ovis* was not isolated. Arrows indicate the breeding periods.

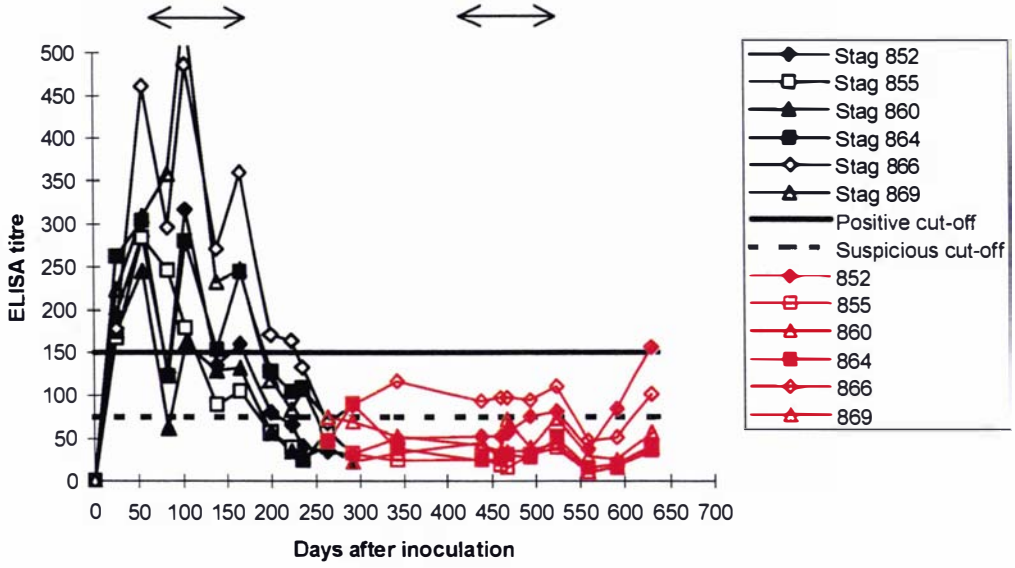


Figure 5.9 *B. ovis* ELISA titres for six stags intravenously inoculated on Day 0 that stopped shedding the organism in semen between 263 and 342 days after inoculation. A black symbol denotes that *B. ovis* was isolated from the semen of the stag while a red symbol denotes that *B. ovis* was not isolated. Arrows indicate the breeding periods.

Stags infected from mating vaginally-infected hinds

Stags 833, 839 and 850 became infected with *B. ovis* by mating vaginally-infected hinds, simulating a "natural" route of infection compared with intravenous inoculation. On Day 134, *B. ovis* was no longer isolated from the semen of stag 833, and on Day 253 *B. ovis* was no longer isolated from the semen of stags 839 and 850 (Table 5.2). Sera from these stags were positive or suspicious in the CFT and ELISA until Day 77. After Day 77, with the exception of a single serum sample from stag 839 that was positive in the CFT on day 202, sera from all three stags were negative in the CFT (Figures 5.10, 5.11). From day 349 to 542, sera from stag 833 were intermittently suspicious in the ELISA. From Day 77 to 504, sera from stags 839 and 850 were negative in the ELISA and on Day 542 serum from stag 839 was negative in the ELISA while serum from stag 850 was suspicious in the ELISA (Figure 5.11).

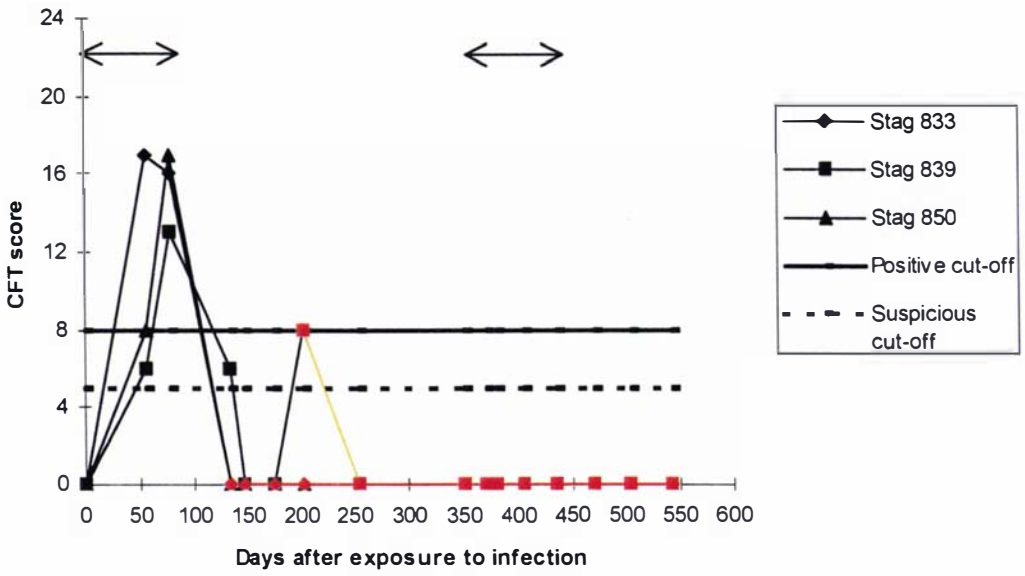


Figure 5.10 *B. ovis* CFT scores for three stags that became infected by mating vaginally-infected hinds about Day 0. A black symbol denotes that *B. ovis* was isolated from the semen of the stag while a red symbol denotes that *B. ovis* was not isolated. Arrows indicate the breeding periods.

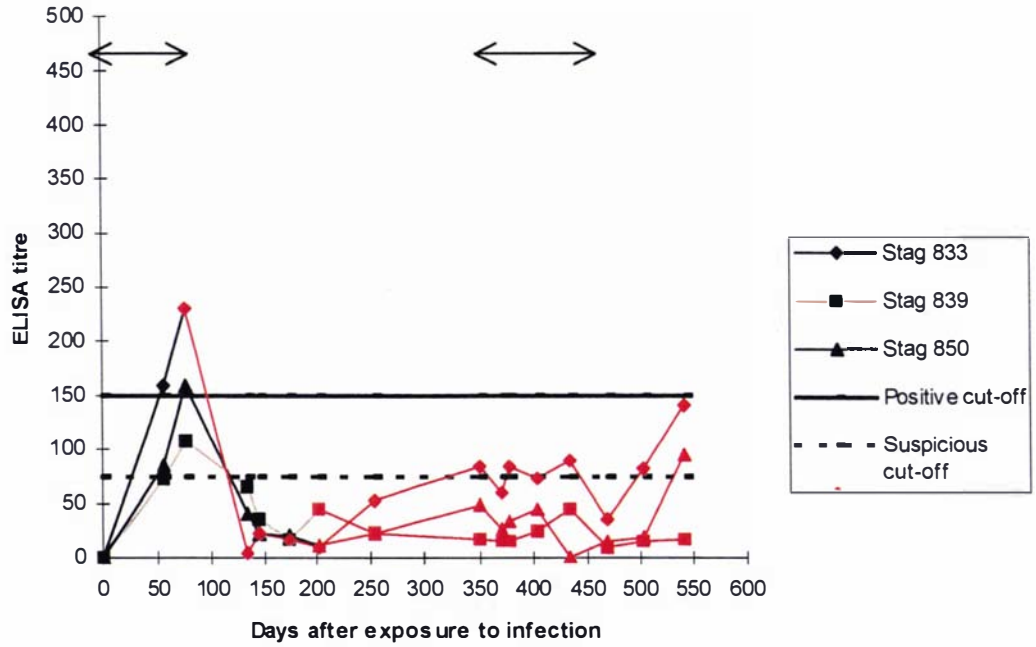


Figure 5.11 *B. ovis* ELISA titres for three stags that became infected by mating vaginally-infected hinds about Day 0. A black symbol denotes that *B. ovis* was isolated from the semen of the stag while a red symbol denotes that *B. ovis* was not isolated. Arrows indicate the breeding periods.

Stags and rams from which B. ovis was not isolated from semen at any sampling time

Two stags (numbers 826 and 861) and two rams (numbers 55 and 56) did not have *B. ovis* isolated from semen at any time during the sampling period (Tables 5.1, 5.3). Sera from the stags were positive in the CFT until Day 83 and were then negative until Day 630. In the ELISA, sera from these two stags were positive or suspicious until Day 166 and then negative until Day 630 (Figures 5.12, 5.13).

Sera from ram 56 were positive in the CFT and ELISA throughout the 630 day experimental period whereas sera from ram 55 were suspicious or negative in both tests from Day 55 until Day 630 (Figures 5.12, 5.13).

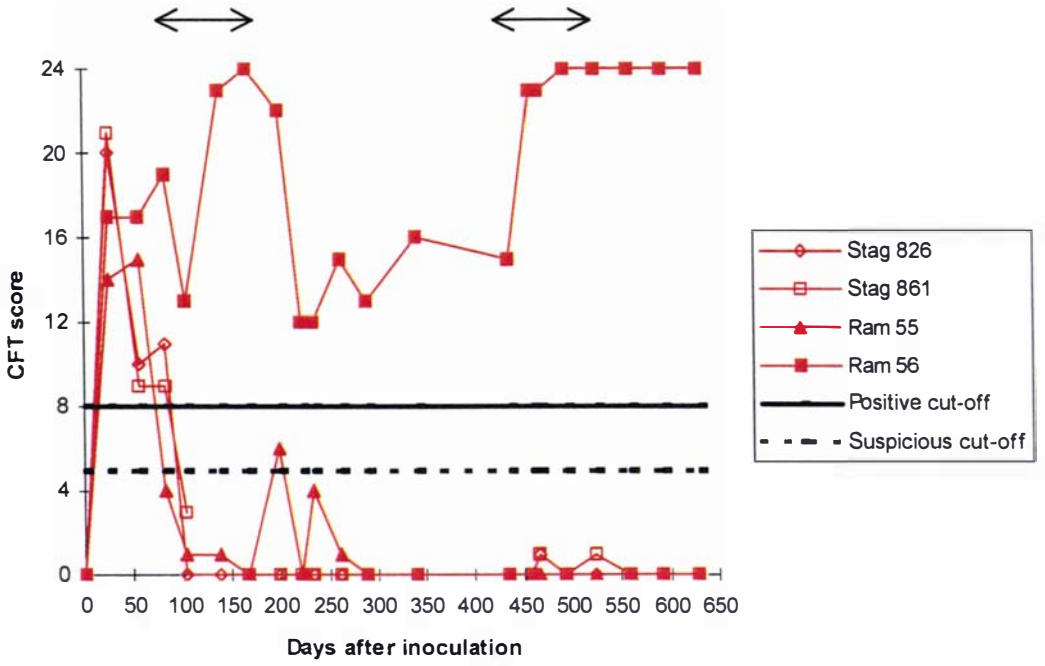


Figure 5.12 *B. ovis* CFT scores for two stags and two rams intravenously inoculated on Day 0 that did not have *B. ovis* isolated from semen at any sampling time. Arrows indicate the breeding periods.

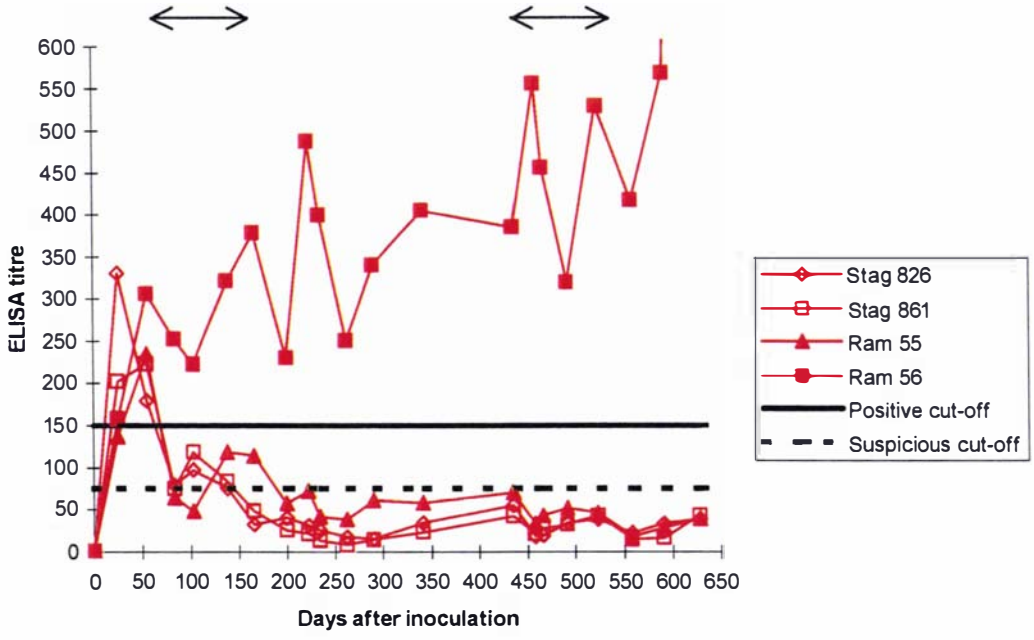


Figure 5.12 *B. ovis* ELISA titres for two stags and two rams intravenously inoculated on Day 0 that did not have *B. ovis* isolated from semen at any sampling time. Arrows indicate the breeding periods.

Using the cut-off values described for sheep (Section 2.5), the sensitivity of the CFT at detecting infected stags in the first 100 days of infection was 100% (n=15 stags; Figure 5.14). From Day 103 to 166, the sensitivity was 80% or better (n=12 to 14 stags), dropping to 30 to 65% from days 200 to 291 (n=6 to 11 stags). The sensitivity of the ELISA was 100% on Day 83 (n=15 stags; Figure 5.14), 75 to 85% from days 103 to 166 (n=12 to 14 stags) and between 10 and 55% from days 200 to 291 (n=6 to 11 stags). On day 342 two of the stags were still shedding *B. ovis* in semen but sera from both of these stags were negative in the CFT and ELISA (Figure 5.14) and it is inappropriate to draw conclusions about test sensitivity from this number of stags. From day 459 to day 630, sera from these 2 stags were consistently positive or suspicious in the CFT and/or ELISA (Figure 5.14).

As an individual test, the ELISA was less sensitive than the CFT but it identified one additional infected stag on day 200, three on day 235 and one on day 263 that were not identified in the CFT. Interpreting both the CFT and ELISA in parallel, the sensitivity of both tests in the first 100 days of infection was 100% (n=15 stags; Figure 5.14). From day 103 to 166 the sensitivity was 80% or greater (n=12 to 14 stags), dropping to 50 to 75% from days 200 to 291 (n=6 to 11 stags).

In contrast, the sensitivity of the CFT and ELISA at detecting infection in the rams was 100% throughout the experiment with the exception of day 630 when serum from one ram (number 59) was negative in the CFT but positive in the ELISA (Figure 5.15).

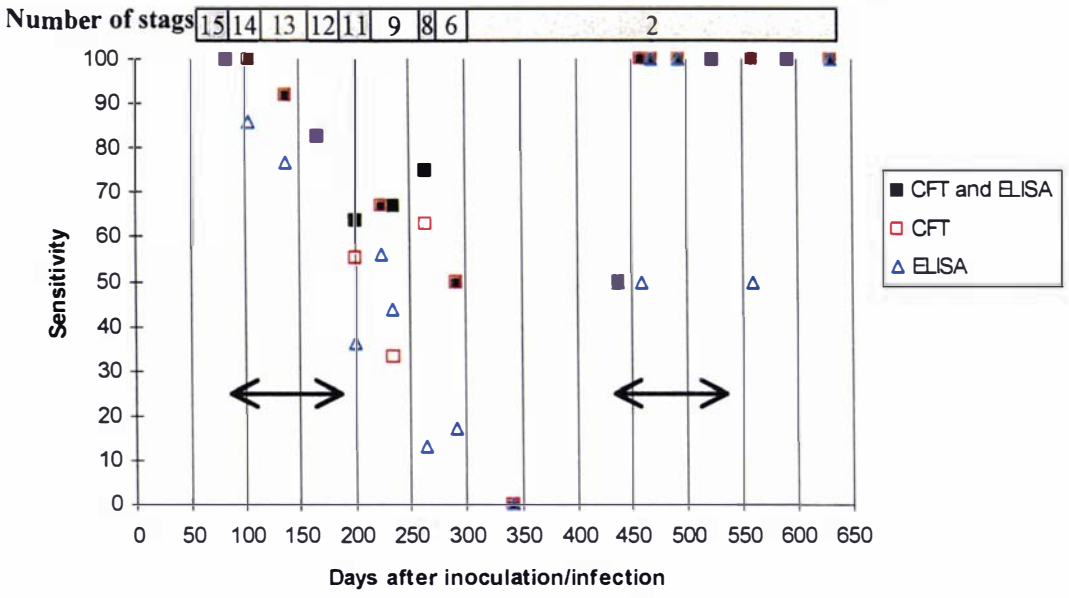


Figure 5.14 Sensitivity of the *B. ovis* CFT, ELISA, and CFT and ELISA read in parallel when used to test sera from 15 stags artificially infected with *B. ovis* on Day 0 that shed the organism in semen for variable lengths of time after infection. Arrows indicate the breeding periods.

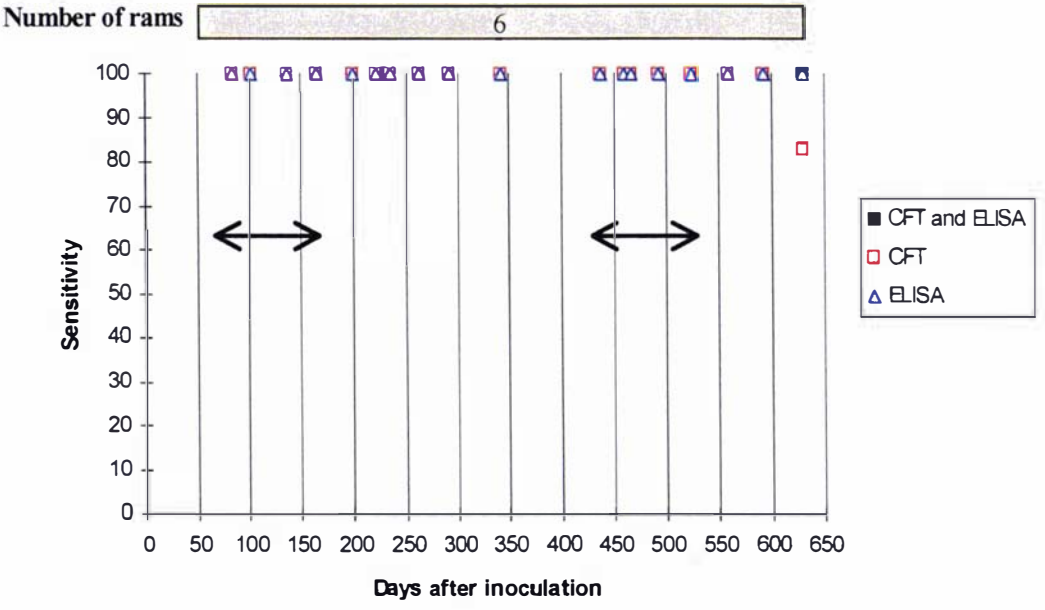


Figure 5.15 Sensitivity of the *B. ovis* CFT, ELISA, and CFT and ELISA read in parallel when used to test sera from six rams artificially infected with *B. ovis* on Day 0 that shed the organism in semen throughout the experiment. Arrows indicate the breeding periods.

5.3.3 Pathology

Gross pathology

Group A stags

On Day 83 after inoculation and throughout the experimental period, two of the 14 Group A stags had lesions of epididymitis that could be detected by scrotal palpation and these lesions were grossly visible following slaughter on Day 649. Stag 854, which stopped shedding *B. ovis* in semen between days 166 and 200, had enlargement of the head, body and tail of the right epididymis with corresponding atrophy of the right testes (Figure 5.16). On incision, the epididymes and testes appeared normal. Stag 864, which stopped shedding *B. ovis* in semen between days 263 and 291, had a mild enlargement of the tail of the right epididymis which on incision appeared normal. During the experimental period, no lesions were detected during scrotal palpation of the remaining 12 Group A stags and there were no other obvious gross lesions in the epididymes of these stags at slaughter on Day 649. At slaughter, there were no visible lesions in the seminal vesicles or ampullae of any of the Group A stags.

Group B stags

During the experimental period, no lesions were detected during scrotal palpation of the Group B stags. At slaughter approximately 561 days after becoming infected by mating vaginally-infected hinds stag 833, which stopped shedding *B. ovis* in semen between Days 77 and 134, had an adhesion between the tunica vaginalis and the tail of the epididymis on the right hand side, but the epididymes appeared otherwise normal. There were no gross lesions in the epididymes of stags 839 or 850, and no gross lesions in the seminal vesicles or ampullae of any of the Group B stags.

Rams

On Day 83 after inoculation and throughout the experimental period, six of the eight rams had lesions of epididymitis that could be detected by scrotal palpation, and these lesions were visible at slaughter on Day 649. These six rams shed *B. ovis* throughout the sampling period and the organism was isolated from their reproductive tracts at slaughter (Tables 5.3, 5.4).

Of the infected rams, number 57 had mild enlargement of the tail of the right epididymis. Ram 58 had enlargement of the tails of both epididymes, particularly on the right hand side. There was also enlargement of the head and body of the epididymis on the right hand side, thickening of the tunica vaginalis and adhesions between the tunic and the testes and epididymes. Ram 59 had a mild enlargement of the tail of the left epididymis, with a firm 10mm nodule on the ventro-caudal aspect containing tan fluid. Ram 60 had enlargement of the tail of the right epididymis with a 10mm nodule on the ventral aspect containing white-tan inspissated material. Ram 61 had enlargement of the head, body and tail of the epididymis on the right hand side with corresponding testicular atrophy, and enlargement of the tail of the left epididymis. The tunica vaginalis was thickened with multiple adhesions between the tunic and the testes and epididymes. A 20mm nodule filled with green-tan inspissated material was present in the ventral aspect of the right epididymal head (Figure 5.1.17). Ram 69 had gross enlargement of the tail of the left epididymis with thickening of the tunica vaginalis. The epididymes of the two rams that did not shed *B. ovis* in their semen (numbers 55 and 56) felt normal on scrotal palpation and appeared grossly normal. The seminal vesicles and ampullae of all rams appeared normal.

Histopathology

All 14 Group A stags, six of eight rams that were artificially infected by intravenous inoculation, and all three Group B stags that became infected by mating vaginally infected hinds had histological lesions in the epididymes, seminal vesicles and/or ampullae consistent with, or suggestive of, *B. ovis* infection (Table 5.5). In the epididymes, lesions consisted of spermatic granulomas, intra-epithelial cysts and/or infiltration of lymphocytes into the lamina propria. In the seminal vesicles and ampullae, lesions consisted of lymphocytic infiltration into the lamina propria and inflammatory cells in the tubules. In all stags, the lesions consisted of a mild increase in the number of cells or one to four discrete foci of cells, each containing 10 to 20 lymphocytes. In contrast the lesions in the rams were more severe, consisting of a marked increase in the number of cells or large coalescing aggregations of lymphocytes. There were no histological lesions in the epididymes, seminal vesicles or ampullae of the two rams that did not shed *B. ovis* in their semen (numbers 55 and 56) or eight stags whose reproductive tracts were collected for comparative purposes.

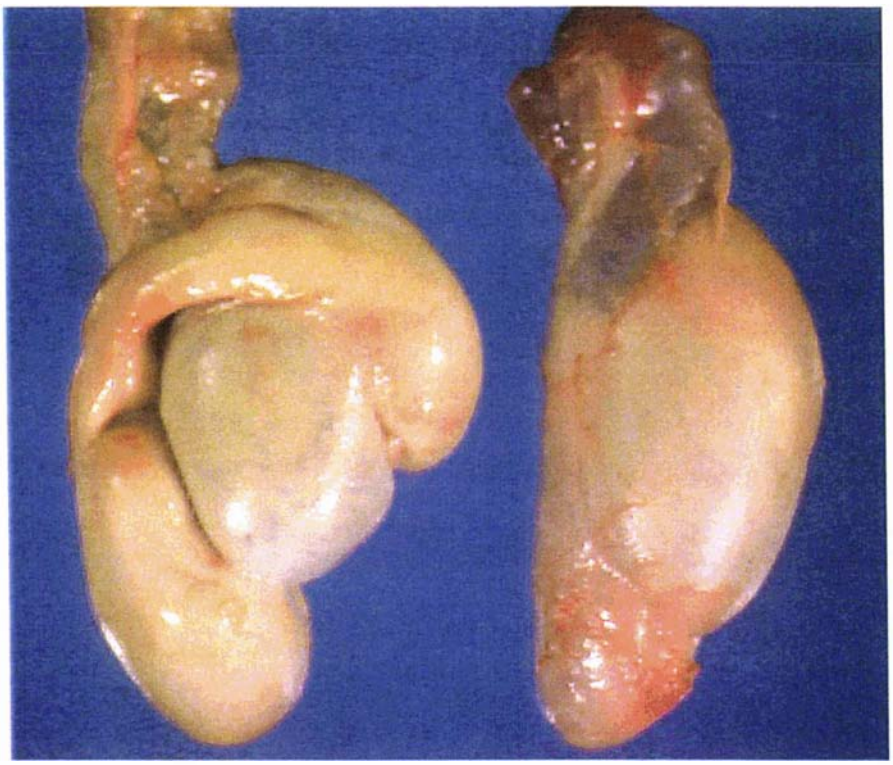


Figure 5.16 Enlargement of the head, body and tail of the right epididymis of stag 854.

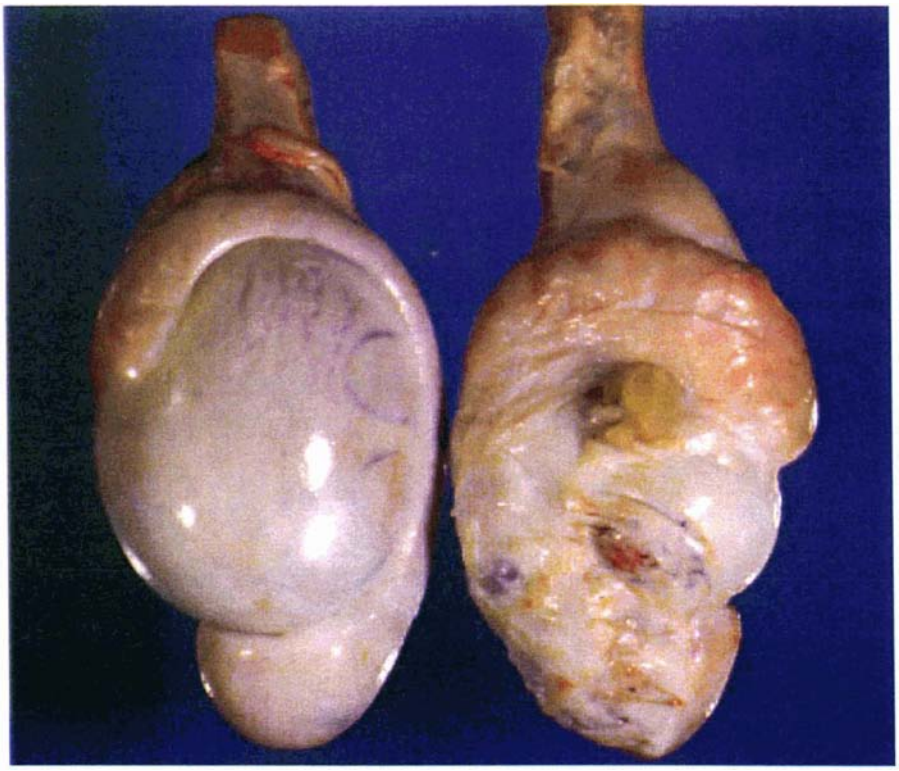


Figure 5.17 Enlargement of the tails of both epididymes and of the right epididymal body and head of ram 61.

Table 5.5 Presence of histological lesions in the epididymes, seminal vesicles and ampullae of 17 stags and eight rams infected with *B. ovis* on Day 0 and slaughtered on Day 649 (Group A stags) or Day 561 (Group B stags).

Species	ID	Infection status	Epididymes	Seminal vesicles	Ampullae
Cervine	**803**	Positive	+	+	sus
Group A	**827**	Positive	+	NS	NS
Cervine	**842**	Negative d263	sus	sus	sus
Group A	**852**	Negative d342	-	sus	-
	854	Negative d200	+	sus	+
	855	Negative d342	-	sus	-
	860	Negative d342	-	+	sus
	864	Negative d291	-	-	sus
	865	Negative d223	+	sus	+
	866	Negative d342	+	sus	+
	867	Negative d138	-	-	sus
	869	Negative d291	+	+	+
Cervine	**826**	Never shed	+	-	-
Group A	**861**	Never shed	sus	sus	+
Cervine	**833**	Negative d134	-	sus	sus
Group B	**839**	Negative d253	+	-	+
	850	Negative d253	sus	sus	sus
Ovine	**57**	Positive	+	+	+
	58	Positive	+	+	+
	59	Positive	+	+	+
	60	Positive	+	+	+
	61	Positive	+	-	-
	69	Positive	+	+	+
Ovine	**55**	Never shed	-	-	-
	56	Never shed	-	-	-

Infection status - Positive: *B. ovis* isolated from semen/reproductive tract throughout the 649-day infection period

Infection status - Negative: shed *B. ovis* in semen initially, but then stopped. "d" refers to the days after infection at which *B. ovis* was no longer isolated from semen.

Infection status - Never shed: *B. ovis* not isolated from semen at any time during the infection period

+: lesions consistent with *B. ovis* infection

sus: lesions suggestive of *B. ovis* infection

-: no lesions consistent with, or suggestive of, *B. ovis* infection

NS: no sample

5.4 Discussion

This is the first longitudinal investigation of *B. ovis* infection in stags, and a total of 17 stags and eight rams infected with *B. ovis* were monitored. The animals were monitored for 561 or 649 days, with blood and semen samples collected at approximately monthly intervals. The number of animals used and the frequency of sampling allow valid conclusions to be drawn. Fourteen of the stags and all eight rams were infected by intravenous inoculation, and it is possible that the progression of infection may differ in natural infections. However, in this experiment three stags that had been infected by mating vaginally infected hinds showed a similar progression of infection to the 14 intravenously inoculated stags, suggesting that the intravenous infection model is likely to mimic natural infection in most respects.

It would appear that stags are as susceptible to *B. ovis* infection as rams. In this experiment 12 of 14 stags (86%) compared with six of eight rams (75%) artificially inoculated with *B. ovis* developed a patent infection, where the organism was shed in semen for a period of time following inoculation. This result is consistent with previous research where intravenous inoculation of stags has resulted in two of three stags (West et al., 1999) and five of six stags (Section 4.3) developing a patent infection. In rams, other researchers have found intravenous inoculation resulted in patent infection in between 80 to 95% of rams (Table 1.2.1; Buddle, 1953; Jebson et al., 1954; Ris, 1964).

In this experiment *B. ovis* was initially isolated from the semen of 12 intravenously inoculated stags and three stags that became infected by mating vaginally-infected hinds. However, over a period of 103 to 342 days after infection the organism was no longer isolated from the semen of 13 of these 15 stags, nor was it isolated from the epididymes, seminal vesicles or ampullae of these stags at slaughter 561 or 649 days after infection. It seems likely that these stags initially developed a patent *B. ovis* infection with localisation of the organism in the reproductive tract and subsequent shedding of *B. ovis* in the semen, but that over time they resolved the infection. It is unknown whether the remaining two stags would have eventually resolved the infection, or whether they would have continued to shed the organism in semen indefinitely. In contrast, the six rams that developed a patent infection continued to shed the organism

in semen throughout the experiment and it was isolated from the reproductive tract at slaughter 649 days after inoculation. Similarly, while the persistence of infection in rams has not been well defined, other researchers have stated that rams continue to shed *B. ovis* in semen for periods in excess of two to four years (Hartley et al., 1955; Buddle, 1955). The apparent ability of the majority of stags to resolve *B. ovis* infection within a year of infection represents an important difference in the characteristics of the disease in stags compared with rams.

Despite being in contact with other stags that were shedding *B. ovis* in semen during the 649-day trial period (including two stags that shed throughout the trial period), the 13 stags that resolved the infection did not develop a further patent infection. Likewise, the two stags and two rams from which *B. ovis* was never isolated from semen did not become infected over time, despite being in continuous contact with infected animals. This experiment incorporated two mating periods, when transmission of *B. ovis* between stags has previously been demonstrated (West et al., 1999), and it would seem likely that following resolution these 13 stags became immune or resistant to the disease. There was a rise in antibody titre during the second breeding period in sera from eight of the 13 stags that resolved the infection, and it is possible that this was in response to further challenge. This data would also suggest that the optimum time to detect infection or exposure to infection in stags may be during or just after the breeding period.

Regardless of whether stags resolved the infection or continued shedding *B. ovis* throughout the experiment, the serological titre patterns were reasonably consistent. The CFT and ELISA were 100% sensitive at detecting infection for the first 83 days after inoculation or infection but the sensitivity of both tests declined over time. This means that when investigating *B. ovis* infection in a commercial deer herd, serological results must be interpreted with caution because if the herd has been infected for some time there is an increased likelihood of false-negative reactions. This aspect of the infection will be further discussed in Chapter 8.

As an individual test the ELISA had a lower sensitivity than the CFT but on three occasions it identified additional infected stags that were not identified in the CFT,

thereby enhancing the overall sensitivity. Therefore, when testing deer for *B. ovis* infection it would be advisable to use both tests and interpret the results in parallel. In sheep, the ELISA has been reported to have a slightly higher sensitivity than the CFT (Worthington, 1984), but a recognised disadvantage is the interpretation of reactions around the cut-off value (Hilbink, 1993), which was also found to be a problem with sera from the stags used in this experiment. Many of the "false-negative" reactions from stag sera had titres from 40-70, and lowering the cut-off values used for the ELISA may increase the sensitivity. However, it may also result in a corresponding decrease in specificity. The sensitivity and specificity of the CFT and ELISA will be further discussed in Chapter 8.

In contrast with stags, in this experiment the CFT and ELISA were 100% sensitive at identifying infected rams with the exception of day 630 where the CFT failed to detect one ram. There are three published reports of investigation of serological titres of rams over time. Webb et al. (1980) artificially infected 10 rams by preputial inoculation and tested their sera in a CFT at weekly intervals for a year. Nine of the rams maintained positive or suspicious titres whereas sera from the tenth ram fluctuated between positive, suspicious and negative titres throughout the experiment. Burgess and Norris (1982) used a CFT to test sera from three rams artificially infected by intranasal inoculation for a 300-day period. It was stated that all three rams showed a decline in titre over time and serum from one ram gave no reaction on day 297. Worthington et al. (1985) collected blood and semen samples at two to four-weekly intervals for 13 to 14 months from 42 infected rams obtained from *B. ovis* infected flocks. They reported that while infected, rams usually continued to produce antibody that could be detected by the CFT or ELISA. Because actual data were not supplied in these reports they are difficult to interpret but it would appear that in general, infected rams continue to have detectable serum antibody levels for at least one to two years after infection. The long-term persistence of serum antibodies that can be detected by standard serological tests represents another important difference in the characteristics of *B. ovis* infection in stags compared with rams.

Brucella ovis was consistently isolated from the semen of five of the six rams that had developed a patent infection but on three occasions it was not cultured from the semen

of the sixth ram, number 61. *Brucella ovis* was isolated from the semen of this ram at the sampling periods immediately before and after each of these three occasions, a pattern that other researchers have described as "intermittent" shedding (Hughes, 1968; Worthington, 1985). From ram 61, the three semen samples from which *B. ovis* was not isolated contained clear accessory sex gland fluid only and at slaughter, isolation of *B. ovis* and histological lesions occurred only in the epididymes and not the seminal vesicles or ampulla of this ram. This suggests that the organism only localised in the epididymes of this ram and would explain why *B. ovis* was not isolated from semen samples that only contained accessory sex gland fluid.

In this experiment, *B. ovis* was isolated from the urinary bladder of four of six infected rams. This finding has not been reported elsewhere although the organism has been isolated from the kidneys of rams (Table 1.3.2; Biberstein et al., 1965; Shott and Young, 1971; Worthington et al., 1985; Plant et al., 1986). This suggests that infected urine might play a role in the transmission of *B. ovis*.

Only two of 17 stags had lesions of epididymitis that could be detected by scrotal palpation during the experimental period, and these lesions were still present at slaughter on Day 649. Similarly, in a previous experiment (Chapter 4.2), of five stags that became infected by contact with infected rams, only one had enlargement of the epididymes that could be detected by scrotal palpation. This would suggest that palpation of the epididymes through the scrotum, or visualisation of the epididymes at slaughter, is unlikely to be a sensitive indicator of infection in individual stags. However, depending on the prevalence of infection within a herd it may give an indication of whole-herd infection status. In contrast, all six rams that became infected with *B. ovis* had gross enlargement of one or both epididymes, which could be detected by scrotal palpation throughout the infection period. In natural infection in commercial flock rams, Hughes (1968) reported that only 30 of 88 (34%) had detectable lesions of epididymitis and the high prevalence of lesions in these six experimental rams may be due to the dose and route of infection used, or it may be a chance occurrence.

Histological lesions consisting of lymphocytic infiltration into the lamina propria of the epididymes, seminal vesicles and ampullae, and spermatic granulomas and intra-

epithelial cysts in the epididymes were present in all 17 stags and six of the eight rams. These lesions were similar to those described by previous researchers (Kennedy et al., 1956; Biberstein et al., 1964; Barron et al., 1985; Foster et al, 1987). However, the lesions in the reproductive organs of the stags were mild and consisted mainly of either a slight diffuse increase in the number of cells or one to four distinct foci containing 10 to 20 lymphocytes. This contrasted with the lesions seen in the rams, which had severe, coalescing infiltration of lymphocytes and suppurative exudation into the tubules. Marked histological lesions were seen in the epididymes, seminal vesicles and ampullae of five stags that were slaughtered within one to two months of infection (Chapter 4.2; Figures 4.2.8, 4.2.9, 4.2.10, 4.2.11). These five stags had large spermatic granulomas in the epididymes, and marked infiltration of lymphocytes into the lamina propria of all three organs. This would suggest that in stags, cellular changes in the reproductive tract in response to *B. ovis* infection might regress over time and this may be a function of resolution of the infection. However at slaughter the lesions present in the epididymes, seminal vesicles and ampullae of stag 803, and the lesions in the epididymes of stag 827, both of which were still shedding *B. ovis* in the semen, did not appear significantly more severe than lesions present in stags that had resolved the infection.

When undertaking histopathological examination, it was difficult to validate lesions that were "positive" and "negative" for *B. ovis*. However, for comparative purposes the reproductive tracts of eight non-infected stags were examined, with no evidence of lymphocytes in the lamina propria of the epididymes, seminal vesicles and ampullae, or intra-epithelial cysts and spermatic granulomas in the epididymes. If these lesions are considered suggestive of *B. ovis* infection, from the histopathological evidence it would appear possible that stags 826 and 861, from which *B. ovis* was not isolated on any occasion, did at some stage have localisation or contact of the organism with the reproductive tract. Both of these stags had histological lesions in the epididymes, seminal vesicles and/or ampullae consistent with, or suggestive of, *B. ovis* infection. It is possible that *B. ovis* localised in these organs but these two stags resolved the infection prior to day 83, the day on which the first semen sample was collected for *B. ovis* culture. This is consistent with the results from six stags artificially infected by inoculation of mucous membranes (Section 4.5) which did not have *B. ovis* isolated

from the semen or reproductive tract but had histological lesions present in the reproductive tract consistent with *B. ovis* infection.

In contrast, there were no histological lesions observed in the epididymes, seminal vesicles or ampullae of the two rams from which *B. ovis* was not isolated (rams 55 and 56), suggesting they overcame the infection before it localised in the reproductive tract. Similarly, Plant et al. (1986) examined the reproductive tracts of 27 rams that were artificially inoculated with *B. ovis* and developed serum antibodies but did not shed the organism in semen, and found no evidence of histological lesions in the reproductive tracts of any of the rams.

5.5 Conclusions

1) Stags are as susceptible to *B. ovis* infection by intravenous inoculation as rams.

2) The majority of stags resolve *B. ovis* infection within a year of becoming infected. In contrast, rams remain infected for periods in excess of 21 months.

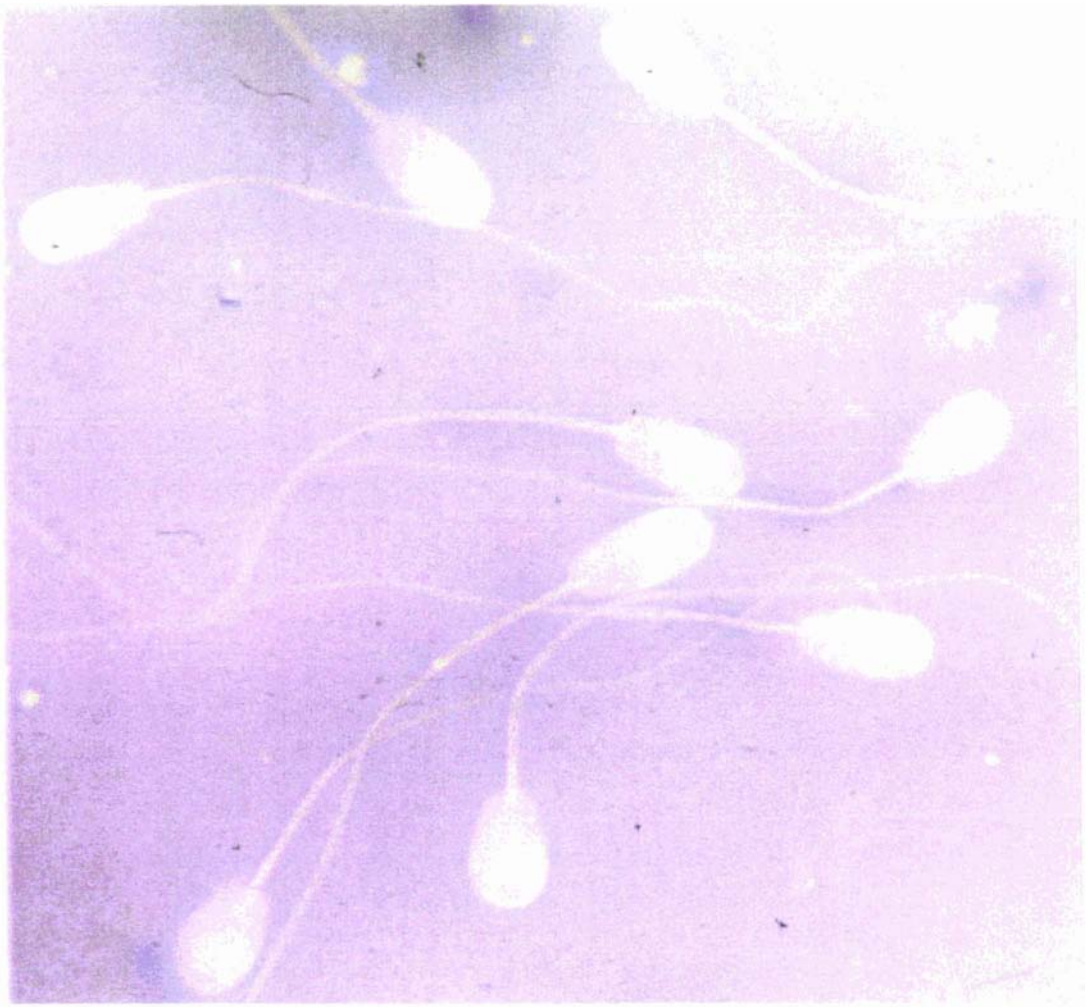
3) Stags develop serum antibodies that are detectable by commercial *B. ovis* CFT and ELISA tests used for sheep. However, the sensitivity of the tests decreases as the time from infection increases. In contrast, during long-term infection of rams the sensitivity of the tests remains high.

4) Detection of epididymal enlargement by scrotal palpation is an insensitive test for diagnosis of *B. ovis* in stags.

5) Histological lesions were present in the epididymes, seminal vesicles and ampullae of stags infected for a 649-day period, but the lesions were milder than those seen in the reproductive organs of rams that had been infected for the same time period.

Chapter Six

Effects of *Brucella ovis* infection on the semen characteristics of stags



Aspects of this chapter, entitled "The effects of *Brucella ovis* infection on the semen characteristics of 16-month-old red deer stags" by AL Ridler and DM West, have been published in the New Zealand Veterinary Journal, 50, 19-22, 2002.

6.1 Introduction

Brucella ovis infection of rams results in reduced sperm motility and an increased number of abnormal sperm, the majority of which are detached sperm heads (Cameron et al., 1971; Cameron and Lauerman, 1976; Kimberling et al., 1986; Kott et al., 1988). Infection has been associated with an increase in cellular and sperm debris in semen (Jebson et al., 1954; Jebson et al., 1955; Webb et al., 1980) and there has been some indication that it may cause a decrease in sperm output (Cameron and Lauerman, 1976).

The effects of *B. ovis* infection on the semen characteristics of stags are largely unknown. Nevertheless, the first recognition of *B. ovis* infection in deer in New Zealand occurred when a semen sample was collected from a rising-3-year old stag for artificial breeding purposes. The semen was visibly purulent and, on microscopic examination, it contained leukocytes but no sperm (Bailey, 1997), suggesting that the infection had detrimental effects on the stag's semen. It is not known whether the semen characteristics of infected stags change over time. The majority of infected stags have been demonstrated to stop shedding *B. ovis* in semen within a year of becoming infected (Chapter 5) but it is unknown whether this results in parallel changes in semen characteristics.

Therefore the objectives of this experiment were to assess the effects of *B. ovis* infection on semen characteristics of stags, and compare the semen characteristics of infected stags while shedding *B. ovis* in semen and following cessation of shedding.

6.2 Materials and methods

General sample collection and analysis methods are included in Chapter 2. A timeline of the series of events in this experiment is summarised in Figure 6.1

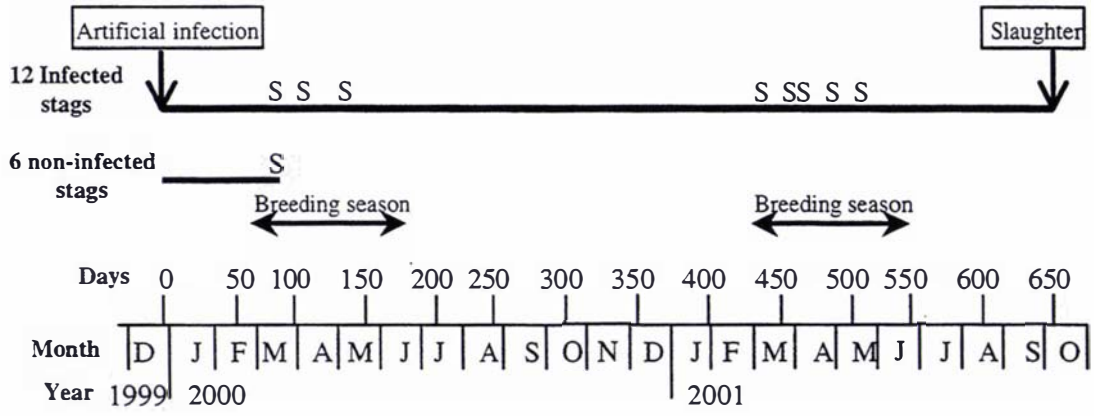


Figure 6.1 Timeline of events for an experiment investigating the effects of *B. ovis* infection on the semen quality of red deer stags. S = semen samples collected for evaluation.

6.2.1 Animals

Twelve red deer stags that had been artificially infected with *B. ovis* on 23 December 1999 by intravenous inoculation (Chapter 5) and six non-infected control stags of the same age, size and peer group were used. After December 23 1999 the two groups were managed similarly, but in separate paddocks to avoid transmission of the organism.

6.2.2 Semen collection

On March 16 2000, 83 days after inoculation, semen was collected from both the infected and non-infected stags. At this time all stags were approximately 15 months old and all 12 of the artificially infected stags were shedding *B. ovis* in semen.

On April 4 and May 5 2000, 103 and 138 days after inoculation, semen was collected from the infected stags only. On Day 138, stag 867 had stopped shedding *B. ovis* in the semen but the other 11 stags were still shedding the organism (Chapter 5; Table 5.1).

During the following breeding season on March 5, March 27, April 4, April 30 and May 31 2001 (Days 437, 459, 467, 493 and 524 after inoculation), semen was collected from the artificially infected stags. At these sampling times 10 of the 12 stags had ceased shedding *B. ovis,* with the organism not isolated from their semen for at least 100 days, while the remaining two stags were still shedding the organism (Chapter 5; Table 5.1). At these sampling times the stags were 27 to 29 months old.

6.2.3 Semen evaluation

Gross semen evaluation

Semen was evaluated visually for colour and the presence of purulent material, urine or gel-like accessory sex gland fractions.

Sperm motility

Immediately after collection, semen was diluted in phosphate buffered saline at 36.8°C and examined under a cover-slip at 400x magnification using phase-contrast microscopy. The percentage of sperm showing progressive forward motility was visually estimated.

Sperm morphology

At the time of collection, one drop of undiluted semen was mixed with one drop of eosin-nigrosin stain (Society for Theriogenology, Hastings, Nebraska, USA) and a smear made on a microscope slide. One hundred sperm were randomly examined under oil immersion at 1000x magnification and assessed for morphological abnormalities. Abnormal heads, abnormal detached heads, proximal droplets, midpiece abnormalities, coiled tails, and looped tails with a droplet enclosed were grouped as "major" abnormalities. Dag defects, defined as sperm with folding and coiling of the midpiece with the axis of the main fold in the distal half of the midpiece (Barth and Oko, 1989) were also classified as major abnormalities. Detached heads, simple looped tails,

terminal coiled tails, abaxial tails, distal droplets and fractured necks were grouped as "minor" abnormalities, as described by Blom (1983).

Leukocyte assessment

At the time of collection, a smear of the semen was made on a microscope slide and later stained with Diff-Quik (Dade Behring Inc, Newark, DA, USA). The relative number of leukocytes was determined by randomly selecting 10 sites on the semen smear and examining them at 1000x magnification. The average number of leukocytes seen per high power field (hpf) was graded using a numbering system where 0 equated to no white cells seen, 1 equated to 1-5 leukocytes/hpf, 2 equated to 5-10 leukocytes/hpf and 3 equated to greater than 10 leukocytes/hpf.

6.2.4 Statistical analysis

Data for sperm motility and morphology were transformed using an arc-sine transformation. For normally distributed data, comparisons between infected and non-infected animals were made with a Student's two-tailed t-test using Microsoft Excel version 9.0 (Microsoft Corp.). For data that were not normally distributed, comparisons between the two groups were made with a Mann-Whitney test using SPSS version 9.0 (SPSS Inc., Chicago, IL, USA). Variation in the motility and morphology of sperm from semen collected from each individual stag in either the 2000 breeding season or the 2001 breeding season were analysed with a general linear model using SAS version 8.2 (SAS Institute Inc., Cary, NC, USA). Results where the p-value was less than 0.05 were considered statistically significant.

6.3 Results

6.3.1 Comparison of semen characteristics between infected and non-infected 15-month old stags

Gross semen evaluation

Semen from seven of the 12 stags shedding *B. ovis* contained purulent material that was grossly visible as either small flecks or large clots (Figure 6.2). There was no purulent material visible in the semen from any of the non-infected stags.

Sperm motility

The progressive forward motility of sperm in semen collected from the 12 infected stags ranged from 0 to 80% with a mean of 27.5%. The motility of sperm in semen from eight of the 12 infected stags was equal to or less than 25% (Table 6.1). The progressive forward motility of sperm in semen collected from the six non-infected stags ranged from 50 to 90% with a mean of 74.2%, and was significantly higher ($p = 0.002$) than the sperm motility in semen from the infected stags (Table 6.1).

Sperm morphology

A range of morphological abnormalities were identified in sperm from both infected and non-infected stags and there were no significant differences between the percentage of normal sperm or percentage of major or minor sperm abnormalities between the infected and non-infected stags (Table 6.1).

Semen from four infected and one non-infected stag had greater than 25% of sperm with major morphological abnormalities (Table 6.1). Of these, semen from two infected stags, numbers 855 and 865 respectively, had 34% and 44% of sperm with proximal droplets. Semen from stag 860 had 70% of sperm with major morphological abnormalities consisting of abnormal heads and proximal droplets, and semen from stag 860 had 33% of sperm with major abnormalities consisting of coiled tails and abnormal heads. Semen from a non-infected stag, number 841, had 30% of sperm with proximal droplets.

Semen from five infected stags had greater than 25% of sperm with minor morphological abnormalities, the majority of which consisted of detached sperm heads (Table 6.1; Figure 6.3). Semen from infected stags contained a significantly higher proportion of sperm with detached heads compared with semen from non-infected stags ($p = 0.006$). Semen from two non-infected stags also had greater than 25% of sperm with minor morphological abnormalities. Semen from stag 841 had 30% minor abnormalities, consisting of 18% detached sperm heads and 12% looped and coiled tails. Semen from stag 850 had 69% of sperm with minor morphological abnormalities, consisting of looped tails.

Presence of leukocytes

Eleven of the 12 infected stags had leukocytes and cellular debris in the semen. Seven stags had greater than 10 leukocytes per high power field, and semen from all of these stags appeared grossly purulent. Three stags had five to 10 leukocytes per high power field, one had less than five and one stag, number 842, had no leukocytes present in the semen (Table 6.1). Many of the leukocytes were too degenerate to classify, but neutrophils and lymphocytes were the main types present. Leukocytes were not present in semen collected from any of the non-infected stags (Table 6.1).

Table 6.1 Sperm motility, percentage of normal sperm, percentage of sperm with major morphological abnormalities, percentage of sperm with minor morphological abnormalities, percentage of sperm with detached heads, sperm density per ml of semen and white cell score in semen from twelve 16-month-old stags artificially infected with *B. ovis* 83 days previously, and in semen from six non-infected stags of the same age.

Stag infection status	Stag ID	% motile sperm	% normal sperm	% major morph. abnorm.	% minor morph. abnorm.	% sperm with detached heads	Sperm density/ml x10^6	White cell score
Infected; shedding *B. ovis* in semen	803	50	79	3	12	12	304.2	2
	827	0	3	7	88	88	26.6	3
	842	80	76	9	6	3	252.5	0
	852	0	47	21	27	20	1.7	3
	854	50	71	9	12	10	184.4	2
	855	0	22	48	23	21	1.8	3
	860	5	25	70	17	15	12.0	3
	864	20	54	8	25	23	497.1	3
	865	25	45	50	7	1	64.0	2
	866	20	37	33	35	20	4.1	3
	867	60	86	8	6	2	1400.0	1
	869	20	12	12	64	56	74.7	3
	Mean	**27.5**	**46.5**	**23.2**	**26.8**	**22.6**	**255.5**	**2.3**
	St dev	**26.7**	**27.7**	**21.8**	**25.3**	**24.9**	**410.8**	**1.0**
Non-infected	831	85	91	3	6	0	221.4	0
	833	75	95	1	4	0	185.4	0
	839	90	84	5	11	0	656.1	0
	841	50	40	30	30	18	10.0	0
	850	60	20	11	69	0	272.2	0
	858	80	98	0	2	5	1132.0	0
	Mean	**74.2**[a]	**71.3**	**8.3**	**20.3**	**3.8**[a]	**412.9**	**0**
	St Dev	**15.9**	**33.1**	**11.3**	**26.0**	**7.1**	**411.0**	**0**

a: statistically significant difference between infected and non-infected stags, $p<0.05$
White cell score: 0 = none seen, 1 = 1-5/hpf, 2 = 5-10/hpf, 3 = >10/hpf
St dev: standard deviation

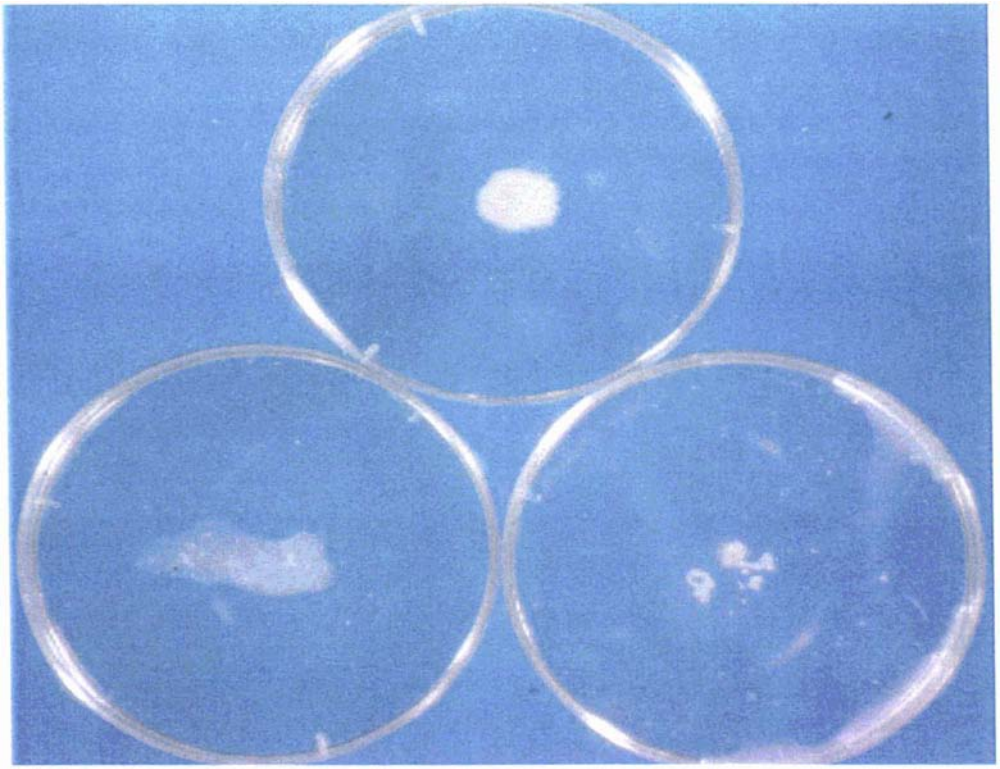


Figure 6.2 Purulent material in the semen of stag 803 (bottom left) and stag 855 (bottom right) that had been artificially infected with *B. ovis* 83 days previously, compared with normal semen from a non-infected stag, number 858 (top)

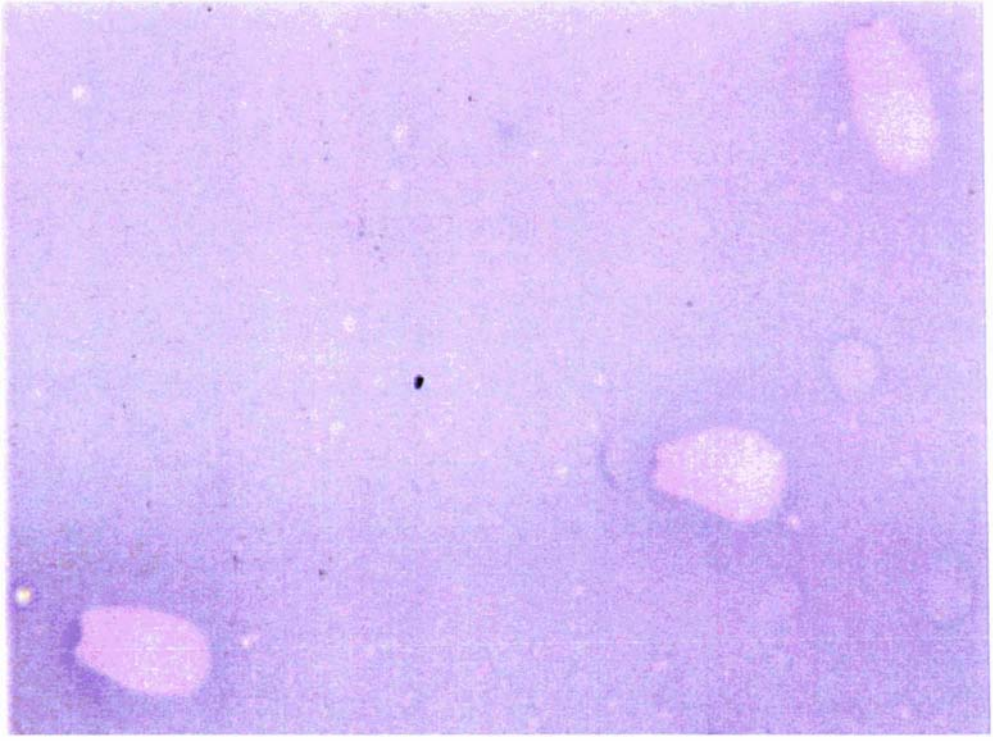


Figure 6.3 Detached sperm heads in a smear of semen collected from stag 827.

6.3.2 Comparison of semen characteristics from artificially infected stags shedding *B. ovis* in semen collected in 2000, and following resolution of infection in 2001

Gross semen evaluation

During 2000, purulent material was present in the semen from eight of the 12 infected stags at one or more sampling times. However, during 2001 no purulent material was visible in the semen collected from any of the 10 stags that had ceased shedding *B. ovis* in semen.

Stags 803 and 827 that shed *B. ovis* in semen throughout the experiment had purulent material in semen during the 2000 sampling period. During the 2001 sampling period, purulent material was present in three of four semen samples collected from stag 803 and in one of three semen samples collected from stag 827.

Sperm motility

For each individual stag, there was no significant difference in the progressive forward motility of sperm between the three semen samples collected in 2000, or between the five semen samples collected in 2001.

Semen from seven of the 10 stags that ceased shedding *B. ovis* prior to the 2001 sampling period showed a significant improvement in the progressive forward motility of sperm following resolution of infection (Table 6.2). Semen from the remaining three stags, numbers 842, 854 and 867, did not show a significant improvement in sperm motility, but semen from these stags had the highest sperm motility of the group in the 2000 sampling period.

Of the two stags that were still shedding *B. ovis* in semen during the 2001 sampling period, semen from stag 803 had a significant decrease ($p=0.01$) in sperm motility whereas semen from stag 827 had a significant improvement in sperm motility ($p=0.04$) (Table 6.2).

Table 6.2 The progressive forward motility of sperm in semen from stags collected in March, April and May 2000, 83 to 138 days after artificial infection with *B. ovis*, and in March, April and May 2001, 437 to 524 days after artificial infection with *B. ovis*, and the mean and standard deviation of progressive forward motility of sperm in semen collected from each stag during the 2000 and 2001 breeding seasons.

		PERCENTAGE OF SPERM WITH PROGRESSIVE FORWARD MOTILITY									
		2000				2001					
Stag ID	Infection status	Mar 16 Day 83	Apr 4 Day 103	May 5 Day 138	Mean (St dev)	Mar 5 Day 437	Mar 27 Day 459	Apr 4 Day 467	Apr 30 Day 493	May 31 Day 524	Mean (St dev)
803	Shedding	50	NS	50	**50 (0.0)**	0	30	NS	5	0	**9[b] (12.4)**
827	Shedding	0	0	NS	**0 (0.0)**	80	70	NS	NS	80	**77[a] (4.7)**
842	Resolved d263	80	70	70	**73 (5.8)**	NS	90	NS	75	60	**75[ns] (12.2)**
852	Resolved d342	0	5	NS	**3 (3.5)**	80	85	80	NS	NS	**82[a] (2.4)**
854	Resolved d200	50	70	40	**53 (15.3)**	60	90	80	NS	NS	**77[ns] (12.5)**
855	Resolved d342	0	20	NS	**10 (14.1)**	70	NS	NS	70	85	**75[a] (7.1)**
860	Resolved d342	5	20	20	**15 (8.7)**	NS	95	NS	80	75	**83[a] (8.5)**
864	Resolved d291	20	10	15	**15 (5.0)**	60	90	60	70	90	**74[a] (13.6)**
865	Resolved d223	25	50	35	**37 (12.6)**	80	70	NS	90	70	**78[a] (8.3)**
866	Resolved d342	20	NS	0	**10 (14.1)**	NS	NS	85	90	60	**78[a] (13.1)**
867	Resolved d138	60	70	70	**67 (5.8)**	80	60	90	90	85	**81[ns] (11.1)**
869	Resolved d291	20	0	10	**10 (10.0)**	80	85	NS	90	80	**84[a] (4.1)**

NS = no sample
a = significant increase in sperm motility in 2001 compared with 2000, $p<0.05$
b = significant decrease in sperm motility in 2001 compared with 2000, $p<0.05$
ns = no significant change in sperm motility in 2001 compared with 2000, $p>0.05$

Sperm morphology

For each individual stag, there was no significant difference in the percentage of normal and abnormal sperm between the three semen samples collected in 2000, or between the five semen samples collected in 2001.

Semen from seven of the 10 stags that ceased shedding *B. ovis* following the 2000 sampling period had a significant decrease in the proportion of morphologically abnormal sperm and a concurrent increase in the proportion of normal sperm in the 2001 sampling period (stags 852, 855, 860, 864, 865, 866, 869; Table 6.3).

Between the 2000 and 2001 sampling periods, semen from stags 852, 855, 866 and 869 showed a significant decrease in the number of sperm with both minor and major abnormalities (Tables 6.4, 6.5). The decrease in the number of major abnormalities in semen from stag 855 was due to a decrease in the number of sperm with proximal droplet, while semen from stags 852 and 866 showed a decrease in the number of sperm with abnormal sperm heads and sperm with a looped tail enclosing a cytoplasmic droplet. The decrease in the number of minor abnormalities in sperm from all of these stags was due predominantly to a decrease in the number of detached sperm heads (Table 6.6).

Semen from stags 860 and 865 had a significant decrease in the number of sperm with major abnormalities in 2001 compared with 2000 (Table 6.4), which was due to a decrease in the number of sperm with proximal droplets. Semen from stag 864 had a significant decrease in the number of sperm with minor abnormalities, due mostly to a decrease in the number of detached sperm heads (Tables 6.5, 6.6).

Of the two stags that were still shedding *B. ovis* in semen at the 2001 sampling period, semen from stag 827 had significantly fewer ($p=0.0001$) abnormal sperm compared with the 2000 sampling period. This was due exclusively to a decrease in the number of sperm with detached heads (Tables 6.3, 6.5, 6.6). There was no change in the number of normal sperm in the semen from stag 803 between 2000 and 2001 (Table 6.3).

Table 6.3 The percentage of morphologically normal sperm in semen from stags evaluated in March, April and May 2000, 83 to 138 days after artificial infection with *B. ovis*, and in March, April and May 2001, 437 to 524 days after artificial infection with *B. ovis*, and the mean and standard deviation percentage of normal sperm in semen from each stag in the 2000 and 2001 sampling periods.

		PERCENTAGE OF NORMAL SPERM									
		2000				2001					
Stag ID	Infection status	Mar 16 Day 83	Apr 4 Day 103	May 5 Day 138	Mean (St dev)	Mar 5 Day 437	Mar 27 Day 459	Apr 4 Day 467	Apr 30 Day 493	May 31 Day 524	Mean (St dev)
803	Shedding	85	NS	73	**79 (8.5)**	75	82	76	77	67	**75^{ns} (5.4)**
827	Shedding	5	0	NS	**2.5 (3.5)**	NS	91	89	87	70	**84^{a} (9.6)**
842	Resolved d263	85	61	82	**76 (13.1)**	NS	89	NS	NS	93	**91^{ns} (2.8)**
852	Resolved d342	52	32	42	**42 (10.0)**	92	94	89	94	NS	**92^{a} (2.4)**
854	Resolved d200	79	86	47	**71 (20.8)**	82	94	NS	98	NS	**91^{ns} (8.3)**
855	Resolved d342	29	15	NS	**22 (9.9)**	90	NS	NS	98	95	**94^{a} (4.0)**
860	Resolved d342	13	26	36	**25 (11.5)**	95	96	NS	92	89	**93^{a} (3.2)**
864	Resolved d291	67	40	56	**54 (13.6)**	90	96	82	89	76	**87^{a} (7.7)**
865	Resolved d223	43	38	55	**45 (8.7)**	91	NS	95	91	89	**92^{a} (2.5)**
866	Resolved d342	31	42	24	**32 (9.1)**	90	88	82	69	91	**84^{a} (9.1)**
867	Resolved d138	86	91	80	**86 (5.5)**	76	93	94	79	92	**87^{ns} (8.6)**
869	Resolved d291	24	0	14	**13 (12.1)**	80	NS	85	86	91	**86^{a} (4.5)**

NS = no sample
a = significant increase in percentage of normal sperm in 2001 compared with 2000, $p<0.05$
ns = no significant difference in percentage of normal sperm in 2001 compared with 2000, $p>0.05$

Table 6.4 The percentage of sperm with major morphological abnormalities in semen from stags evaluated in March, April and May 2000, 83 to 138 days after artificial infection with *B. ovis*, and in March, April and May 2001, 437 to 524 days after artificial infection with *B. ovis*, and the mean and standard deviation percentage of major morphological abnormalities of sperm in semen from each stag in the 2000 and 2001 sampling periods.

		PERCENTAGE OF SPERM WITH MAJOR MORPHOLOGICAL ABNORMALITIES									
		2000				2001					
Stag ID	Infection status	Mar 16 Day 83	Apr 4 Day 103	May 5 Day 138	Mean (St dev)	Mar 5 Day 437	Mar 27 Day 459	Apr 4 Day 467	Apr 30 Day 493	May 31 Day 524	Mean (St dev)
803	Shedding	3	NS	13	**8 (7.1)**	19	6	5	2	2	**7^{ns} (7.0)**
827	Shedding	7	5	NS	**6 (1.4)**	NS	6	0	3	3	**3^{ns} (2.4)**
842	Resolved d263	9	13	14	**12 (2.6)**	NS	3	NS	NS	6	**5^{ns} (2.1)**
852	Resolved d342	21	22	24	**22 (1.5)**	11	1	1	NS	NS	**4^{a} (5.8)**
854	Resolved d200	9	5	17	**10 (6.1)**	0	3	NS	0	NS	**1^{ns} (1.7)**
855	Resolved d342	48	59	NS	**54 (7.8)**	4	NS	NS	7	3	**5^{a} (2.1)**
860	Resolved d342	70	66	38	**58 (17.4)**	3	4	NS	4	9	**5^{a} (2.7)**
864	Resolved d291	8	29	13	**17 (11.0)**	7	3	12	29	13	**13^{ns} (9.9)**
865	Resolved d223	50	35	37	**41 (8.1)**	17	NS	5	11	11	**11^{a} (4.9)**
866	Resolved d342	33	33	24	**30 (5.2)**	1	6	6	0	7	**4^{a} (3.2)**
867	Resolved d138	8	8	37	**18 (16.7)**	3	5	4	8	7	**5^{ns} (2.1)**
869	Resolved d291	12	20	14	**15 (4.2)**	3	NS	10	4	5	**6^{a} (3.1)**

NS = no sample
a = significant decrease in percentage of sperm with major morphological abnormalities in 2001 compared with 2000, $p<0.05$
ns = no significant difference in percentage of sperm with major morphological abnormalites in 2001 compared with 2000, $p>0.05$

Table 6.5 The percentage of sperm with minor morphological abnormalities in semen from stags evaluated in March, April and May 2000, 83 to 138 days after artificial infection with *B. ovis*, and in March, April and May 2001, 437 to 524 days after artificial infection with *B. ovis*, and the mean and standard deviation percentage of minor morphological abnormalities of sperm in semen from each stag in the 2000 and 2001 sampling periods.

		PERCENTAGE OF SPERM WITH MINOR MORPHOLOGICAL ABNORMALITIES										
		2000					2001					
Stag ID	Infection status	Mar 16 Day 83	Apr 4 Day 103	May 5 Day 138	Mean (St dev)		Mar 5 Day 437	Mar 27 Day 459	Apr 4 Day 467	Apr 30 Day 493	May 31 Day 524	Mean (St dev)	
803	Shedding	12	NS	14	**13**	**(1.4)**	6	12	19	21	31	**18^{ns}**	**(9.5)**
827	Shedding	88	95	NS	**92**	**(4.9)**	NS	5	9	10	27	**13^{a}**	**(9.7)**
842	Resolved d263	6	26	4	**12**	**(12.2)**	NS	3	NS	NS	1	**2^{ns}**	**(1.4)**
852	Resolved d342	27	36	34	**32**	**(4.7)**	11	5	10	6	NS	**8^{a}**	**(2.9)**
854	Resolved d200	12	9	36	**19**	**(14.8)**	10	3	NS	2	NS	**5^{ns}**	**(4.4)**
855	Resolved d342	23	26	NS	**25**	**(2.1)**	1	NS	NS	1	2	**1^{a}**	**(0.6)**
860	Resolved d342	17	8	26	**17**	**(9.0)**	2	0	NS	7	2	**3^{ns}**	**(3.0)**
864	Resolved d291	25	31	31	**29**	**(3.5)**	3	9	6	2	11	**6^{a}**	**(3.8)**
865	Resolved d223	7	27	8	**14**	**(11.3)**	7	NS	0	10	0	**4^{ns}**	**(5.1)**
866	Resolved d342	35	25	45	**35**	**(10.0)**	19	6	9	14	2	**10^{a}**	**(6.7)**
867	Resolved d138	6	1	5	**4**	**(2.6)**	1	2	2	7	1	**3^{ns}**	**(2.5)**
869	Resolved d291	64	80	70	**71**	**(8.1)**	5	NS	5	3	4	**4^{a}**	**(1.0)**

NS = no sample
a = significant decrease in percentage of sperm with minor morphological abnormalities in 2001 compared with 2000, $p<0.05$
ns = no significant difference in percentage of sperm with minor morphological abnormalites in 2001 compared with 2000, $p>0.05$

Table 6.6 The percentage of sperm with detached heads in semen from stags evaluated in March, April and May 2000, 83 to 138 days after artificial infection with *B. ovis*, and in March, April and May 2001, 437 to 524 days after artificial infection with *B. ovis*, and the mean and standard deviation percentage of sperm with detached heads in semen from each stag in the 2000 and 2001 sampling periods.

		PERCENTAGE OF SPERM WITH DETACHED HEADS										
		2000					2001					
Stag ID	Infection status	Mar 16 Day 83	Apr 4 Day 103	May 5 Day 138	Mean (St dev)		Mar 5 Day 437	Mar 27 Day 459	Apr 4 Day 467	Apr 30 Day 493	May 31 Day 524	Mean (St dev)
803	Shedding	12	NS	13	**13**	**(0.7)**	4	12	14	19	29	**16^{ns}** **(9.2)**
827	Shedding	88	95	NS	**92**	**(4.9)**	NS	4	9	10	27	**13^{a}** **(10.0)**
842	Resolved d263	3	7	2	**4**	**(2.6)**	2	2	NS	NS	1	**2^{ns}** **(0.6)**
852	Resolved d342	20	28	30	**26**	**(5.3)**	4	4	6	1	NS	**4^{a}** **(2.1)**
854	Resolved d200	10	6	25	**14**	**(10.0)**	8	2	NS	1	NS	**4^{ns}** **(3.6)**
855	Resolved d342	21	25	NS	**23**	**(2.8)**	1	NS	NS	1	2	**1^{ns}** **(0.6)**
860	Resolved d342	15	6	26	**16**	**(10.0)**	2	0	NS	6	0	**2^{ns}** **(2.8)**
864	Resolved d291	23	26	28	**26**	**(2.5)**	3	7	0	0	11	**4^{a}** **(4.8)**
865	Resolved d223	1	0	4	**2**	**(2.1)**	1	NS	0	0	0	**0^{ns}** **(0.5)**
866	Resolved d342	20	20	32	**24**	**(6.9)**	11	6	7	12	2	**8^{a}** **(4.0)**
867	Resolved d138	2	1	3	**2**	**(1.0)**	1	1	1	6	0	**2^{ns}** **(2.4)**
869	Resolved d291	56	80	68	**68**	**(12.0)**	5	NS	5	3	2	**4^{a}** **(1.5)**

NS = no sample
a = significant decrease in percentage of sperm with detached heads in 2001 compared with 2000, $p<0.05$
ns = no significant difference in percentage of sperm with detached heads in 2001 compared with 2000, $p>0.05$

Relative number of leukocytes

Leukocytes were present in the semen of all infected stags in 2000, at which time all 12 stags were shedding *B. ovis* in semen. In 2001, 10 of the 12 stags had ceased shedding *B. ovis* in semen, but between one and 10 leukocytes per high power field were still present in smears of semen from these 10 stags (Table 6.7).

Stags 803 and 827, which shed *B. ovis* in semen throughout the experiment, had large numbers of leukocytes present in semen during both the 2000 and 2001 sampling periods (Table 6.7).

Table 6.7 The relative number of leukocytes in semen from stags evaluated in March, April and May 2000, 83 to 138 days after artificial infection with *B. ovis*, and in March, April and May 2001, 437 to 524 days after artificial infection with *B. ovis*, and the median relative number of leukocytes in semen from each stag in the 2000 and 2001 sampling periods.

		LEUKOCYTE SCORE									
		2000				2001					
Stag ID	**Infection status**	**Mar 16 Day 83**	**Apr 4 Day 103**	**May 5 Day 138**	**Median**	**Mar 5 Day 437**	**Mar 27 Day 459**	**Apr 4 Day 467**	**Apr 30 Day 493**	**May 31 Day 524**	**Median**
803	Shedding	2	3	NS	**2.5**	2	3	3	3	3	**3**
827	Shedding	3	3	3	**3**	NS	3	3	NS	3	**3**
842	Resolved d263	0	0	1	**0**	NS	1	NS	0	0	**0**
852	Resolved d342	3	NS	2	**2.5**	1	1	0	2	NS	**1**
854	Resolved d200	2	2	2	**2**	1	1	NS	1	NS	**1**
855	Resolved d342	3	NS	3	**3**	2	NS	NS	0	1	**1**
860	Resolved d342	3	1	1	**1**	NS	1	NS	2	0	**1**
864	Resolved d291	3	1	1	**1**	2	1	1	2	1	**1**
865	Resolved d223	2	1	0	**1**	1	1	NS	1	0	**1**
866	Resolved d342	3	3	3	**3**	2	NS	1	1	2	**1.5**
867	Resolved d138	1	1	0	**1**	NS	NS	2	2	1	**2**
869	Resolved d291	3	NS	3	**3**	1	NS	NS	1	1	**1**

NS = no sample
Leukocyte score: 0 = no leukocytes seen, 1 = 1 to 5 leukocytes/hpf, 2 = 5 to 10 leukocytes/hpf, 3 = >10 leukocytes/hpf

6.4 Discussion

This is the first investigation that has been undertaken of the effects of *B. ovis* infection on the semen characteristics of stags. The results from this experiment indicate that *B. ovis* infection has a detrimental effect on the semen characteristics of stags. Compared with non-infected stags, semen from the majority of infected stags had a large number of leukocytes and cellular debris, reduced sperm motility and a large number of sperm with detached heads. These effects on semen characteristics are similar to those seen in rams with *B. ovis* infection (Cameron and Lauerman, 1976; Kott, 1988).

During this experiment, semen was collected by electro-ejaculation rather than artificial vagina because the latter collection method was not logistically feasible. For the semen characteristics considered, electro-ejaculation is considered a valid collection method because it has been demonstrated in rams and bulls that semen collected by electro-ejaculation has similar sperm motility and morphology to that collected by artificial vagina (Austin et al., 1961; Mattner and Voglmayr, 1962; Salamon and Morrant, 1963). However, it has been reported that semen collected from rams and bulls by electro-ejaculation has a lower sperm density to that collected by artificial vagina (Austin et al., 1961; Mattner and Vogylmayr, 1962) and in stags, semen collected by electro-ejaculation is reported to show considerable variation in volume and concentration (Haigh et al., 1985; Hunter, 1997). Sperm density was not considered as a parameter in this experiment.

During this experiment there were no significant differences in the number of normal sperm between the infected and non-infected stags. However, only one semen sample was collected from infected and non-infected stags for comparative purposes, and this sample was collected during March which is at the beginning of the breeding period for red deer in New Zealand. Young stags come into peak sexual activity later in the breeding season compared with mature stags, and they are more likely to exhibit variation in semen characteristics than mature animals (Lincoln and Guinness, 1973). Semen collected from stags at the beginning of the breeding season has been demonstrated to have a higher number of sperm with morphological abnormalities (Haigh et al., 1984; Haigh et al., 1985). At the time of sampling, the stags were only 15

months of age and semen was collected from only six non-infected stags, two of which had a large number of morphologically abnormal sperm. Thus one non-infected stag (number 850) had 69% of sperm with looped tails, a defect which can be artificially induced after semen collection by cold shock or hypo-osmolality of the sperm morphology stain (Barth and Oko, 1989). Hence it is possible that these sperm defects may have been artefactual. A second non-infected stag (number 841) had 30% of sperm with proximal droplets, a defect may have been a reflection of the relative immaturity of this stag and the time of the season at which sampling occurred (Haigh et al., 1985; Barth and Oko, 1989). In retrospect, it would have been useful to collect further semen samples from the non-infected stags for comparative purposes, and it is possible that further sampling would have resulted in a difference between the number of normal sperm from infected and non-infected stags. Similarly, during the period of shedding *B. ovis* in semen four of the infected stags in this experiment had a large number of sperm with proximal droplets or abnormal heads that were not present in 2001 once shedding of the organism had ceased. It is possible that the relative immaturity of the pubertal stags used in this experiment was the cause of these sperm abnormalities rather than an effect of *B. ovis* infection. Sperm with proximal droplets and abnormal heads have not been reported in semen from rams infected with *B. ovis*.

During the 2000 breeding season, two or three semen samples were collected from each infected stag and during the 2001 breeding season, three to five semen samples were collected from each stag. For each stag there was no significant difference between the semen characteristics in each sample during each breeding season. Because a relatively large number of stags were used and because multiple semen samples were collected for evaluation, these data allow for a good comparison of effects of *B. ovis* infection on the semen characteristics of stags while shedding *B. ovis* in semen and after cessation of shedding.

It is difficult to objectively classify semen "quality" and correlate this with fertility (Clarke et al., 1973), but various classifications have been suggested to describe semen as "very good", "good", "fair" and "poor". To classify the morphology of bull semen, the following criteria have been suggested (Carroll et al., 1963; Howard, 1981):

Classification	% Normal sperm	% motile sperm
Very good	>75	>70
Good	60-74	50-70
Fair	40-59	30-50
Poor	<40	<30

Using these classifications of sperm morphology and motility, semen from one of the 12 infected stags that was shedding *B. ovis* (number 842) was "very good", from three stags (numbers 803, 854 and 867) was "good", from three stags was "fair to poor" and from five stags was "poor". Thus, of the 12 stags that were shedding *B. ovis* in semen, eight produced semen that would not be considered acceptable for breeding.

In contrast, during the 2001 breeding season all of the10 stags which ceased shedding *B. ovis* consistently produced semen that, on the basis of sperm morphology and motility, would be classified as "very good". This further suggests that these 10 stags had effectively "resolved" the infection (as discussed in Chapter 5) and raises the possibility that, if a breeding stag becomes infected with *B. ovis,* it may be feasible to keep it until resolution occurred and thereafter use it for breeding. Clearly, a breeding soundness examination including semen evaluation would be neccessitated in these circumstances. Similarly, chemotherapy of an infected breeding stag to hasten "resolution" of the infection may be indicated. In rams, chemotherapy using a combination of oxytetracycline and dihydrostreptomycin for seven to 21 days resulted in six of six rams and 15 of 18 rams discontinuing shedding of *B. ovis* in semen, suggesting that resolution of the infection had taken place (Kuppuswamy, 1954; Dargatz, 1990). Chemotherapy of infected stags has not been attempted and further experimentation is required to establish whether it would hasten resolution of infection.

It is not known how the effects of *B. ovis* infection on the semen characteristics of stags would affect their fertility. It is probable that the majority of infected stags would have reduced fertility and it is likely that some ejaculates from some infected stags would not be capable of achieving pregnancies. In sheep, despite the effects of infection on the semen characteristics of individual rams, infection often does not result in poor reproductive performance within a flock (Middleberg, 1973). However, in most flocks

in New Zealand it is common practice to mate at a relatively high ratio of 1 ram per 50 ewes, and to mate more than one ram to each group of ewes (Smith and Knight, 1998), which may well mask reduced individual ram fertility due to *B. ovis* infection. In contrast, it is common for some deer farmers to mate a single stag to a group of 80 or more hinds (Wilson et al., 1998). Using such mating management, it is possible that *B. ovis* infection of a stag or stags could result in a dramatic reduction in reproductive performance.

It is unknown why leukocytes were still present in the semen of stags following resolution of infection but it may have been due to an autoimmune reaction to sperm that become exposed to the systemic immune system following damage to the reproductive tract during *B. ovis* infection. Exposure of sperm antigen to the systemic immune system following vasectomisation has been demonstrated to result in the formation of antisperm antibodies in every species studied including mice, rats, guinea pigs, rabbits and humans (Bigazzi et al, 1976; Alexander and Anderson, 1979), and the formation of antisperm antibodies may lead to the development of autoimmune orchitis (Mikuz and Damjanov, 1982). Experimentally induced allergic autoimmune orchitis resulted in polymorphonuclear infiltration into the efferent ducts, epididymis and vas deferens (Tung and Alexander, 1977) and if this occurred it is probable that leukocytes would be present in semen. Alternatively it is possible that *B. ovis* organisms were still present within the reproductive tract and were therefore providing continued antigenic stimulation, but that they were present in such low numbers that they were not detected by semen culture. This latter explanation seems unlikely because following resolution of infection, *B. ovis* was at no time isolated from the semen of any of the stags, nor was it isolated from the reproductive tract of these stags at slaughter (Chapter 5).

During the 2001 breeding season, two stags were still shedding *B. ovis* in semen. One of these stags, number 803, had "poor" sperm morphology and motility while stag 827 had "very good" sperm morphology and motility. The reason for these differences are unknown but it would appear that *B. ovis* infection affects the semen quality of stags to differing degrees. Alternatively, it is possible that stag 827 may have been close to resolving the infection.

6.5 Conclusions

1) In the majority of infected stags, *B. ovis* infection results in the presence of leukocytes and cellular debris in semen, reduced sperm motility and a relatively large number of sperm with detached heads. Purulent material may be visible in the semen.

2) After stags have stopped shedding *B. ovis* in semen, the semen characteristics improve to a level likely to be considered acceptable for breeding. However, small numbers of leukocytes are still present in semen.

3) The effects of *B. ovis* infection on the semen characteristics of stags, and the common management practice of mating only one stag to a group of hinds mean that *B. ovis* has the potential to result in poor reproductive performance within a deer herd, and veterinarians should consider this possibility when investigating cases of poor reproductive performance.

Chapter Seven

Effects of *Brucella ovis* infection on the reproductive performance of hinds, and venereal transmission to stags

A summary of this chapter, entitled "Effects of vaginal *Brucella ovis* infection on the reproductive performance of hinds, and venereal transmission to stags" by AL Ridler, DM West, KJ Stafford, PR Wilson and MG Collett, has been accepted by the New Zealand Veterinary Journal and is in press.

7.1 Introduction

The effect of *B. ovis* infection on the reproductive performance of hinds is largely unknown. In New Zealand, sera were collected from 13 hinds two months after they had been mated to the first infected stag identified in this country (O'Neil, 1996). Sera from 12 hinds were negative in the CFT, ELISA and GDT. Sera from one hind was positive in the CFT and suspicious in the ELISA and GDT while a further sample collected from this hind 24 days later was suspicious in the CFT and negative in the ELISA and GDT. There was no report of abortion or perinatal death in this group of hinds (Scott, 1998).

Brucella ovis infection of ewes has been reported to result in late-term abortions or the birth of weak lambs that die soon after birth (Lawrence, 1961). The pathology, histopathology and microbiology of *B. ovis* infection in ewes have been documented (Hartley, 1961; Osburn and Kennedy, 1966; Ris, 1970) but in most cases ewes were artificially infected during pregnancy (Table 1.4.1) and few field cases of *B. ovis*-induced abortion or perinatal lamb mortality have been documented (McFarlane et al., 1952; Rickard-Bell, 1963; Haughey et al., 1968). Similarly, when ewes were experimentally infected by mating to infected rams, in one experiment two of 25 ewes developed placentitis and aborted (Buddle, 1955) while in another there was no evidence of infection in any of 21 ewes (Hartley et al., 1955). Vaginal inoculation of ewes on the day of mating resulted in three lambs from 27 ewes being born with infected placentae, but the lambs remained viable (Hughes, 1972b). This would suggest that abortion or perinatal lamb loss due to *B. ovis* infection are uncommon manifestations of the disease in ewes.

It is recognised that ewes can act as a mechanical vector in the transmission of *B. ovis* between rams by carrying the organism in the vagina (Hartley et al., 1955; Snowden, 1958) but it has not been reported whether this can occur in deer

The objectives of this experiment were to investigate the effects of *B. ovis* infection on the reproductive performance of hinds and to determine whether stags can become infected with *B. ovis* by mating vaginally infected hinds.

7.2 Materials and methods

General sample collection and analysis methods are described in Chapter 2. A timeline of the events in this experiment is summarised in Figure 7.1.

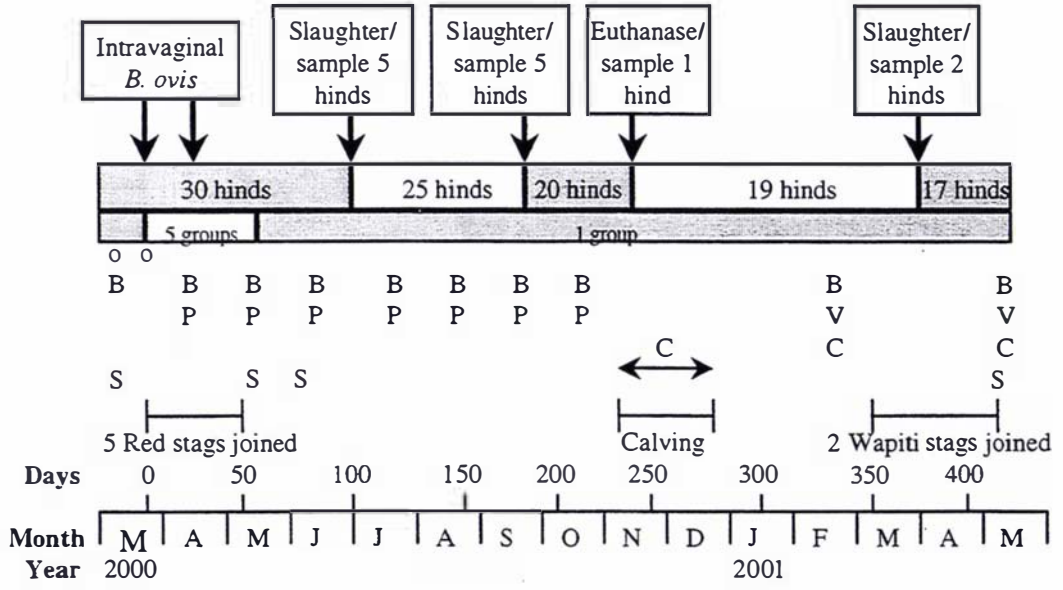


Figure 7.1 Timeline of events for an experiment investigating the effects of vaginal *B. ovis* infection on the reproductive performance of hinds, and venereal transmission to stags. o = oestrus synchronisation, B = blood samples collected from hinds, S = blood and semen samples collected from stags, C = blood samples collected from calves, P = pregnancy scanning hinds, V = vaginal swabs collected from hinds.

7.2.1 Animals

30 mixed-age red deer hinds, with an age distribution ranging from three to 11 years, were used. Seven of the 30 hinds were three-years-old and had not calved at two-years

of age, while the remaining 23 hinds had previously given birth to at least one live calf. Six 15-month-old red deer stags were used for mating.

7.2.2 Hinds

Oestrus synchronisation and mating

On 8 March 2000 (Day -14), 14 days before mating was due to begin, the hinds were synchronised for oestrus by inserting a progesterone releasing device (Eazi-breed CIDR sheep & goat device, Livestock Improvement Corporation, New Zealand) into the vagina. On Day -2 the CIDR was removed and each hind received 200 IU of serum gonadotrophin (Folligon, Pharmaco (NZ) Ltd.) by intra-muscular injection.

On 22 March (Day 0), the expected day of oestrus following synchronisation, 1ml of an inoculum of *B. ovis* was instilled into the anterior vagina of each hind using a sterile pipette. The concentration of this inoculum was estimated on the basis of McFarlane density standards to be approximately $1x10^8$ colony forming units/ml.

Following inoculation, the 30 hinds were randomly allocated into six groups of five hinds and a 16-month-old red deer stag that was serologically negative for *B. ovis* antibodies was joined with each group. The six groups were grazed in separate paddocks and were watched to ensure that animals were not injured and animals from different groups were not mixed. It was noticed that one stag, number 841, stayed separate from his group of hinds and that when he approached the hinds they reacted aggressively towards him. Because this behaviour pattern persisted it was decided at the end of the first oestrous cycle on April 10 (Day 19) to remove this stag from the experiment and join the five hinds that had been with this stag to another mating group containing stag 833 and five hinds. This resulted in five mating groups, one containing a stag and 10 hinds and four containing a stag and five hinds.

At the end of the first oestrous cycle on April 10 (Day 19) each hind received a further intra-vaginal instillation of *B. ovis* inoculum containing $1.2x10^8$ colony-forming units/ml into the anterior vagina. This meant that stags had the potential to be exposed to the *B. ovis* organism a second time by mating hinds that did not conceive in the first

oestrous cycle. The five mating groups were again grazed separately until the completion of the mating period on Day 55, giving a total mating period of three oestrous cycles. At the conclusion of mating the five stags were removed from the mating groups and grazed together as one group and the 30 hinds were grazed together as another group.

Sampling and pregnancy scanning hinds

Blood samples were collected from the hinds at approximately monthly intervals throughout mating and pregnancy on April 10, May 16, June 15, July 20, August 21, September 21 and October 24, 2000 (Days 18, 55, 85, 120, 152, 183, 216), for testing in the CFT and ELISA (Figure 7.1).

On Days 55 and 85, hinds were pregnancy tested by rectal ultrasonography using an Aloka SSD 210DXII ultrasound scanner with a 5MHz linear rectal transducer (Omega Imaging, Cordova, TN, USA). The duration of pregnancies was estimated by measuring the foetal crown-rump length using the method described by Revol and Wilson (1990). On Days 120, 152, 183 and 216 hinds were pregnancy tested by rectal ultrasonography to ascertain whether pregnancy was maintained throughout gestation. In late pregnancy on Days 183 and 216, the pregnancy status of some hinds was assessed based on abdominal contour because it could not be detected by rectal ultrasonography, presumably because the pregnant uterus was in the ventral abdomen and out of range of the ultrasound probe.

A total of 13 hinds were slaughtered during the experiment for both sample collection and production reasons. The samples from hinds, foetuses and calves that were selected for pathological, histopathological and microbiological examination were selected based on results from other researchers investigating experimental infection of ewes (Osburn and Kennedy, 1966; Marco et al., 1994; Grillo et al., 1999).

Four of the 30 hinds did not become pregnant and on Day 97 these four hinds were slaughtered, with the vagina and uterus of each hind collected for histopathology and *B. ovis* culture. On the same day a pregnant hind that was culled for production reasons was slaughtered and the vagina, uterus and foetus from this hind were collected for

histopathology and *B. ovis* culture. Blood samples were collected from all five slaughtered hinds.

On Day 187, 46 days before the start of calving, five hinds that were assessed on the basis of foetal ageing to have conceived on Day 0 were slaughtered. These hinds were slaughtered primarily for sample collection and they were specifically slaughtered as late in pregnancy as possible while still meeting the regulations for transport of pregnant animals. Three of the five hinds selected were considered the most likely to have infected placenta as they had shown the highest serum antibody levels up to that point, while the remaining two hinds were randomly selected. From each hind the uterus, placenta, spleen and foetus were collected for histopathology. Uterine fluid, iliac lymph nodes, spleen and foetus were collected for *B. ovis* culture.

On Day 240, a hind that had an assisted calving of a dead calf two days previously and was suffering from severe laminitis was euthanased. The uterus was collected for histopathology. Uterine fluid, spleen, iliac and mammary lymph nodes were collected for culture. A blood sample for *B. ovis* serology was collected prior to euthanasia.

On Day 378 two hinds, one of which had *B. ovis* isolated from the vagina on Day 337 and one of which had an assisted calving four months previously were slaughtered. The uterus, vagina and spleen were collected for histopathological examination and the uterus, vagina, spleen, iliac and mammary lymph nodes were collected for *B. ovis* culture. A blood sample was also collected from each hind.

On Day 337 blood samples were collected from the 19 remaining hinds and on Day 421 a further blood sample was collected from each of the remaining 17 hinds. At the same time a plain cotton-tipped swab of the anterior vagina was taken for *B. ovis* culture.

7.2.3 Calves

Monitoring of calving

Hinds were due to start calving on Day 233 (November 10, 2000). From Day 222 until Day 280 hinds were monitored every one to two days. Newborn calves were blood

sampled for *B. ovis* CFT and ELISA testing and identified with an ear-tag. The identity of each calf, the dam and the date of birth were recorded.

Sampling of foetuses

A total of six foetuses were collected when their dams were slaughtered. One foetus was collected on Day 97 and based on crown-rump length this foetus was estimated to be approximately 78 days old. A further five foetuses were collected on Day 187 and were estimated to be 187 days old, approximately 46 days from full-term. From all foetuses samples of lung, liver and spleen were collected for histopathological examination. Foetal lung, liver, spleen and abomasal contents were collected for culture and blood samples were collected for serology.

Sampling of dead calves

Three autolysed calves were born by assisted delivery. The degree of autolysis in two of the calves was considered too excessive to make culture worthwhile but lung, liver, spleen and abomasal contents were collected from the freshest calf for *B. ovis* culture. Another calf was born dead and sections of lung, liver and spleen from this calf were examined histologically and lung, liver, spleen and abomasal contents were cultured for *B. ovis*. At post-mortem examination, heart blood was collected from all four dead calves.

Sampling of live calves

Between November 6 and December 29 2000, sixteen calves were born alive. Throughout the calving period hinds were monitored every one to two days and newborn calves were caught, blood sampled and identified with an ear-tag. Two calves were unable to be caught at birth and hence were not blood sampled at this time. One calf died approximately four days after birth but the remainder survived until the end of the experiment on May 17, 2001 when the calves were 22 to 27 weeks of age. Thus 14 calves were blood sampled soon after birth and 15 were blood sampled at weaning on February 22, 2001 when they were 10 to 15 weeks of age and on May 17, 2001 at 22 to 27 weeks of age.

7.2.4 Stags

Sampling of red stags used during the 2000 breeding season

On Days 57 and 77 (May 18 and June 7 2000), two and 22 days after the end of mating, blood samples were collected from the five stags for testing in the CFT and ELISA, and semen samples were collected for *B. ovis* culture.

On Day 97 (June 27) stags 831 and 858 were slaughtered. Blood samples were collected for CFT and ELISA testing, and the epididymes, seminal vesicles and ampullae were collected for *B. ovis* culture. The remaining three stags, numbers 833, 839 and 850, were retained and joined with 14 other artificially infected stags to investigate the long-term persistence and effects of infection in stags (Chapter 5).

2001 breeding season

In the following breeding season, 17 hinds remained and these were mated as a separate group to a seven-year-old Wapiti stag from March 7 to April 15, 2001. This stag was replaced by a nine-year-old Wapiti stag, which was joined with the hinds until May 15 2001. Blood samples were collected from the two Wapiti stags at the conclusion of mating on May 15 2001.

7.3 Results

7.3.1 Hinds

Pregnancy

Over the mating period of three oestrous cycles, 26 of the 30 hinds became pregnant (Table 7.1) and all hinds remained pregnant until either slaughter or parturition.

Table 7.1 Number of hinds impregnated in each of three oestrous cycles by five 14-month-old stags, and number of non-pregnant hinds. The time of impregnation was estimated by measuring the foetal crown-rump length using rectal ultrasonography.

Stag	Total number of hinds	First oestrous cycle	Second oestrous cycle	Third oestrous cycle	Non-pregnant hinds
833	10	4*	4	0	2
839	5	3	1	0	1
850	5	2	0	2	1
831	5	3	1	1	0
858	5	5	-	-	0
TOTAL	**30**	**17**	**6**	**3**	**4**

*Stag 833 was mated to only five hinds in the first oestrous cycle, and a further five hinds were joined with this stag at the beginning of the second oestrous cycle

Serology

On Day 19, all 30 hinds had positive or suspicious antibody titres in one or both of the CFT and ELISA. During the following two months there was a decline in titres with occasional hinds registering a positive or suspicious antibody titre, usually in one test only (Figures 7.2, 7.3). Raw serological data is included in Appendix 1 (Tables A1.12, A1.13).

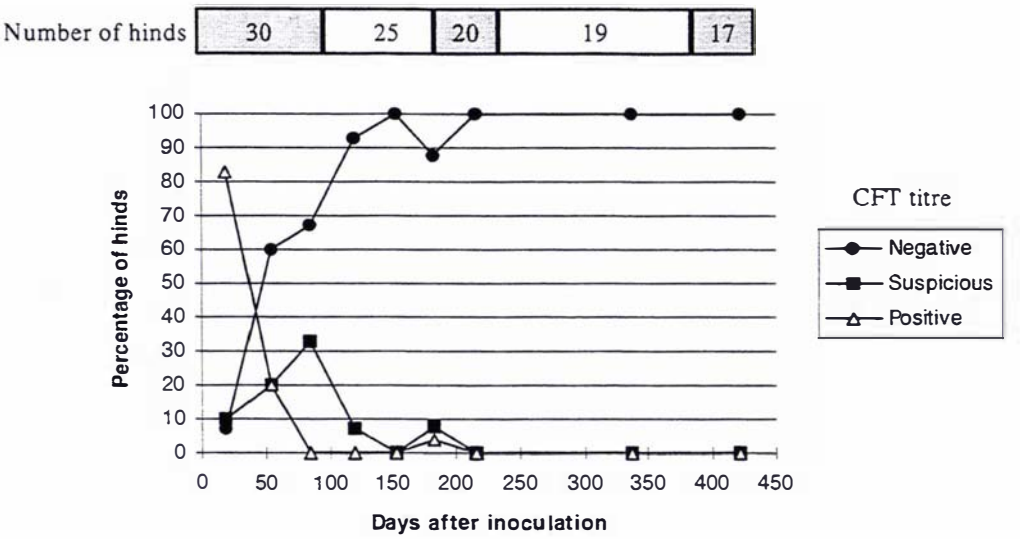


Figure 7.2 The percentage of hinds artificially infected with an intra-vaginal inoculation of *B. ovis* on Days 0 and 19 with positive, suspicious or negative serum *B. ovis* CFT titres at variable times after infection.

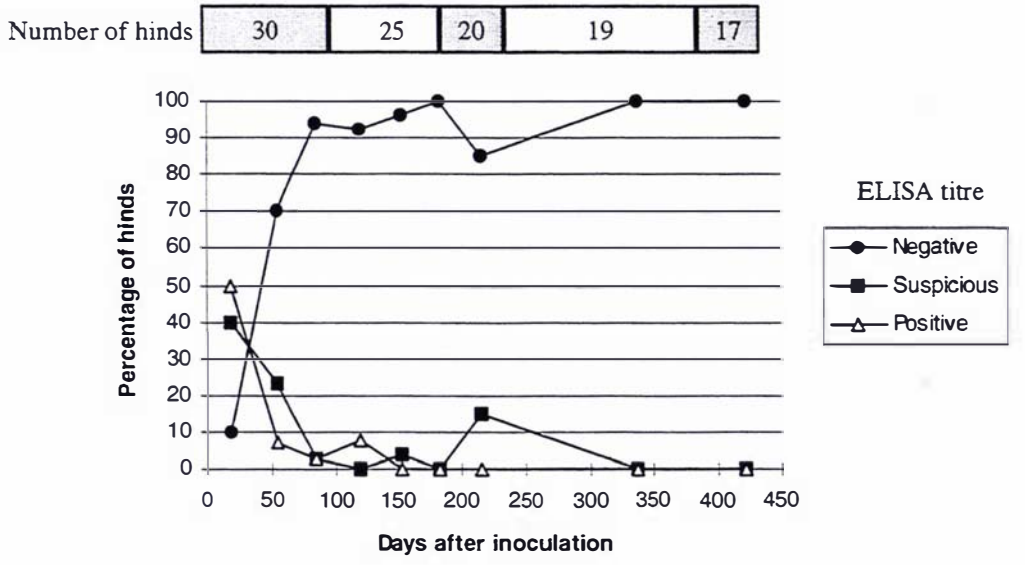


Figure 7.3 The percentage of hinds artificially infected with an intra-vaginal inoculation of *B. ovis* on Days 0 and 19 with positive, suspicious or negative serum *B. ovis* ELISA titres at variable times after infection.

Histopathology and microbiology

No significant gross or histological lesions were seen in any samples examined with the exception of hind 11 (Table 7.2). This hind was assisted in the delivery of a full-term autolysed calf. She also had severe laminitis and was subsequently euthanased. Grossly, a purulent metritis was present and on histological examination a subacute mucopurulent necrotising metritis was seen with Gram negative and Gram positive rods present. Culture of the uterus on blood agar yielded a heavy mixed growth, but culture on *B. ovis* selective media yielded no growth. *Brucella ovis* was not cultured from the other samples collected from slaughtered hinds or foetuses (Table 7.3).

On Day 337 *B. ovis* was isolated from the vagina of hind number 12 but not from the vagina of any of the remaining 18 hinds. Pulsed field gel electrophoresis of this isolate using the method described in Chapter 3 confirmed that it was the same strain inoculated into the vagina of this hind 337 days previously. Hind 12 and another hind (hind 13), which was culled for production reasons, were slaughtered on Day 378 but *B. ovis* was not isolated from the vagina, uterus, spleen, and iliac or mammary lymph nodes of either hind.

Serum collected at slaughter from hind number 13 on Day 378, which had been separated from the hind group and paddocked with hind 12 for 41 days prior to slaughter, was positive in the CFT with a titre of 4/16 and positive in the ELISA with a titre of 259. With the exception of sera collected on Days 19 and 55 after artificial infection, all sera collected from this hind prior to being separated from the hind group had been negative in the CFT and negative in the ELISA with titres of less than 29.

Table 7.2 Histopathological examination of tissues collected from hinds that received an intra-vaginal inoculation of *B. ovis* on Days 0 and 19 and were slaughtered or euthanased at variable times after inoculation.

Hind ID	1-4	5	6-10	11	12, 13
Days since initial artificial infection with *B. ovis*	97	97	187	240	378
Stage of pregnancy	Non-pregnant	Early pregnancy	Late pregnancy	Assisted calving. Metritis	Non-pregnant
Vagina	-	-	NS	NS	-
Uterus	-	-	-	-*	-
Placentome	NS	-	-	-	-
Spleen	NS	NS	-	-	-
Foetal tissues	NS	-	-	NS	NS

-: lesions consistent with, or suggestive of, *B. ovis* infection not present

NS: no sample examined

*: lesions present, but not consistent with, or suggestive of, *B. ovis* infection

Table 7.3 Tissue samples cultured for *B. ovis* from hinds that received an intra-vaginal inoculation of *B. ovis* on Days 0 and 19 and were slaughtered or euthanased at variable times after inoculation.

Hind ID	1-4	5	6-10	11	12, 13
Days since initial artificial infection with *B. ovis*	97	97	187	240	378
Stage of pregnancy	Non-pregnant	Early pregnancy	Late pregnancy	Assisted calving. Metritis	Non-pregnant
Vagina	-	-	NS	-	-
Uterus	-	-	-	-*	-
Placentome	NS	-	-	-	NS
Spleen	NS	NS	-	-	-
Iliac lymph node	NS	NS	-	-	-
Mammary lymph node	NS	NS	NS	-	-
Foetal tissues	NS	-	-	-	NS

-: *B. ovis* not isolated

NS: no sample examined

*: heavy growth of mixed organisms, but *B. ovis* not isolated

Calving

Twenty of the 30 hinds were retained until parturition. Sixteen hinds gave birth to live calves and 15 calves survived until weaning. Three hinds required assistance at calving and were delivered of full-term calves that had been dead for approximately a day. In all cases the calves were presented in an anterior position with the legs out. Moderate traction was required to deliver two of the calves. The head of the third calf was twisted at the neck and a partial foetotomy of the head, combined with strong traction to remove the body, was required. A further hind gave birth to a freshly dead calf and one calf died approximately four days after birth, possibly due to starvation following mis-mothering.

7.3.2 Calves

Histopathology and culture of foetuses and dead calves

The foetuses and dead calves had no gross or histological lesions in the lung, liver or spleen. *Brucella ovis* was not isolated from any of the samples collected from the six foetuses at slaughter, or from samples collected from two dead calves.

Serology of foetuses, dead calves and live calves

Serum collected from foetuses and calves that were born dead were negative in the *B. ovis* CFT and the ELISA. ELISA titres ranged from zero to one.

Sera from 14 live calves sampled at one to three days of age had CFT titres ranging from 0 to 4/32 with a mean of 3/4, and ELISA titres ranging from 12 to 276 with a mean of 141. At weaning when the calves were 10 to 15 weeks of age and again in May when the calves were 22 to 27 weeks of age, the CFT titres were all zero and the ELISA titres were negative, ranging from one to seven with a mean of 2.7 (Table 7.4). Raw serological data is included in Appendix 1 (Table A.1.14).

Table 7.4 The number of calves whose dams were artificially infected with an intra-vaginal inoculation of *B. ovis* immediately before and during the mating period with positive, suspicious or negative serum *B. ovis* CFT and ELISA titres at 1-3 days of age and at 10-15 and 22-27 weeks of age.

Age of calves	1-3 days (n=14)		10-15 and 22-27 weeks (n=15)	
	CFT	**ELISA**	**CFT**	**ELISA**
Negative	10	5	15	15
Suspicious	1	4	0	0
Positive	3	5	0	0

Performance of calves

The average live-weight gain of the 15 surviving calves up to weaning at 10 to 15 weeks of age was 415 grams per day.

7.3.3 Stags

By estimating the conception date of hinds using ultrasonographic foetal ageing, it appeared unlikely that stag 841 impregnated any hinds. Of the remaining five stags, number 833 impregnated eight of 10 hinds, stags 839 and 850 each impregnated four of five hinds and stags 831 and 858 each impregnated five of five hinds (Table 7.1).

On Day 57 after introduction to the hinds, which was two days after the end of mating, serum from stag 833 was positive in the CFT and ELISA, serum from stag 839 was suspicious in the CFT and ELISA and serum from stag 850 was positive in the CFT and suspicious in the ELISA. *Brucella ovis* was cultured from semen collected from these three stags (Table 7.5).

On day 77 after introduction to the hinds, which was 22 days after the end of mating, sera from stags 833 and 850 were positive in the CFT and ELISA and serum from stag 839 was positive in the CFT and suspicious in the ELISA. Again, *B. ovis* was isolated from the semen of all three stags (Table 7.5).

On Days 57 and 77 after introduction to the hinds, and at slaughter on Day 97 sera from Stags 831 and 858 were negative in the *B. ovis* CFT and ELISA and *B. ovis* was not cultured from semen collected from these stags on either of these days (Table 7.5). At slaughter on Day 97, sera from these two stags were negative in the CFT and ELISA and *B. ovis* was not isolated from the epididymes, seminal vesicles or ampullae.

Table 7.5 CFT and ELISA titres from sera collected from five stags 55 and 77 days after introduction to hinds that were artificially infected with *B. ovis* by intra-vaginal inoculation, and isolation of *B. ovis* from the semen of these stags.

	DAY 57			DAY 77		
Stag ID	**CFT titre**	**ELISA titre**	***B. ovis* isolation from semen**	**CFT titre**	**ELISA titre**	***B. ovis* isolation from semen**
833	1/64	159	+	4/32	229	+
839	2/8	75	+	1/32	108	+
850	4/8	86	+	1/64	160	+
831	0	6	-	0	8	-
858	0	9	-	0	6	-

CFT titre interpretation: <4/4 negative; 1/8-3/8 suspicious; >4/8 positive
ELISA titre interpretation: <75 negative; 75-150 suspicious; >150 positive
+: *B. ovis* isolated
-: *B. ovis* not isolated

7.4 Discussion

This is the first reported experiment investigating the effects of *B. ovis* infection on the reproductive performance of hinds and the role that hinds play in the transmission of the disease. In this experiment a total of 30 hinds were used, 10 of which were slaughtered prior to parturition for pathological examination and 20 of which were retained for calving. The hinds were infected with a *B. ovis* culture instilled into the vagina on the day of mating in an attempt to mimic a "natural" infection that would result from a hind being mated by an infected stag. Following inoculation, there was no evidence of foetal loss, abortion or perinatal death that could be attributed to *B. ovis* infection. Furthermore, with the exception of the isolation of *B. ovis* from the vagina of one hind 337 days after inoculation, the organism was not isolated from any of the hind or foetal body tissues selected for examination, nor were gross or histological lesions consistent

with *B. ovis* infection present in any of the organs examined. All hinds developed serum antibody titres within 19 days of inoculation indicating exposure to *B. ovis*, but it appears probable that they overcame the organism before it became localised in the body.

Similar results have been reported in ewes that were infected by a "natural" route, either by mating to infected rams or by vaginal inoculation on the day of mating (Buddle, 1955; Hartley et al., 1955; Hughes, 1972b). It appears that in ewes and hinds, *B. ovis* infection is unlikely to cause economically significant losses due to abortion or perinatal death. It is possible that, as has been demonstrated in ewes, experimental challenge of hinds during pregnancy would result in abortion or perinatal death but this was not investigated in this study. However, it is unlikely that natural infection of hinds or ewes would occur during gestation.

Fifteen hinds gave birth to normal healthy calves that attained weaning weights which were above average compared with other commercial New Zealand red deer calves (Beatson et al., 2000a). It is possible that these calves may have been born with infected placentas but remained viable, which has been reported in lambs born to artificially infected ewes (Hughes, 1972b). Hinds ingest their placenta shortly after calving and therefore placentas from calving hinds were not available for collection. However, placentas from five hinds slaughtered 46 days before parturition were examined with no evidence of *B. ovis* infection.

The development of serum antibody titres in the hinds indicated exposure to the organism, but in most hinds the titres decreased to negative levels within 120 days of the first inoculation of *B. ovis*. Therefore during an investigation of poor pregnancy rates in hinds where poor stag fertility was suspected, serological testing of hinds for *B. ovis* infection may be of value within 120 days of mating. Serological testing of hinds for *B. ovis* would be a particularly valuable investigative option if the stag or stags that were used for mating were not available for testing, for example if the stags were leased or slaughtered after mating.

Sera from the majority of live calves blood sampled at one to three days of age were positive or suspicious in the CFT and/or ELISA suggesting either exposure to the organism or passive transfer of antibody from the dam. It has been demonstrated that the foetal lamb is able to produce *B. ovis* antibodies in response to infection (Biberstein et al., 1966; Tierney et al., 1997) and it is possible that the antibodies present in these calves may have been due to active immunity from exposure to the organism *in-utero.* However it is more likely that the antibodies were due to passive maternal immunity from colostral intake. Prior to calving all hinds had *B. ovis* serum antibodies although, using the serological titre cut-off values described for sheep, these were below "suspicious" levels. Antibodies are concentrated in colostrum and Ris (1970) demonstrated that *B. ovis* antibody titres in the colostrum of infected ewes were much higher than the serum titres of those ewes at that time. Foetuses and calves that had not suckled did not have *B. ovis* serum antibodies, supporting the hypothesis that the serum antibody in live calves was due to maternal antibody. By weaning at 10 to 15 weeks of age there was no evidence of serum antibodies in any of the live calves, suggesting waning of passive maternal immunity. One could argue that *B. ovis* antibody levels in deer do decline over time regardless of infection status (Chapter 5) but in the absence of any other evidence of infection it seems unlikely that these calves had systemic *B. ovis* infection as foetuses. The technical difficulties with collection of colostrum from hinds means that it was not collected for *B. ovis* antibody testing.

Three of five mating stags became infected with *B. ovis* after mating hinds that had been artificially infected by vaginal inoculation, demonstrating venereal transmission of *B. ovis* to stags. This is similar to experimental studies in which rams were infected by mating ewes immediately after they had been mated by an infected ram (Hartley et al., 1955; Snowden, 1958). It cannot be determined why the remaining two stags did not become infected with *B. ovis,* but it may be related to individual variation in susceptibility to disease or varying levels of exposure to the organism. It is unknown how common it is for a hind to be mated by more than one stag during the same oestrus period but if it does occur in mating systems where more than one stag is joined to a group of hinds, then there is the potential for stag to stag transmission by this route.

Brucella ovis was cultured from the vagina of one hind, number 12, 337 days after inoculation. Ewes have been reported to carry the organism in the vagina for at least 64 days (Muhammed et al., 1975). In the absence of any gross or histological changes in the vagina of this hind it would appear likely that the organism was residing there as a commensal. If *B. ovis* has the ability to reside as a commensal organism in the genital tract of some females for long periods of time then this could be a potential complicating factor when attempting to eradicate the disease from a flock or herd.
However *B. ovis* has been successfully eradicated from sheep in the Falkland Islands (Reichel et al., 1994), Flinders and King Islands (Ryan, 1964) and individual flocks in New Zealand (West and Bruce, 1991) by testing and culling rams only, suggesting that persistence of infection in ewes is unlikely to play a significant role in maintenance of infection on a property between years. In the current experiment two Wapiti stags were used for mating the remaining hinds in the 2001 breeding season. Sera collected from these stags at the end of the mating period were negative in the CFT and ELISA, suggesting that these stags did not become infected with *B. ovis*. However, hind 12 was slaughtered prior to the 2001 breeding season and the remaining 17 hinds did not have *B. ovis* isolated from the vagina, so it is unlikely that the Wapiti stags were exposed to the organism.

Brucella ovis was not isolated from the vagina of hind 12 at slaughter on day 378 even though it had been isolated from the vagina of this hind 41 days previously. This may have been because the infection had resolved within those 41 days or due to poor sampling technique. Alternatively it is possible that the organism was residing in the kidney or urinary bladder and was cultured from the vagina on day 337 due to urine contamination. Serum from hind 13, a hind that was separated from the hind group and paddocked with hind 12 for the 41 days before slaughter, was positive in both the CFT and ELISA at slaughter on Day 378. Sera from this hind had been negative in the ELISA and CFT for the previous 323 days, and the increase in titre suggests that this hind had been re-exposed to the organism, possibly by sniffing vaginal discharge or urine from hind 12.

In this experiment there was a high degree of reproductive wastage but this is not considered to be due to *B. ovis* infection. The pregnancy rate of 87% in the 30 hinds is acceptable compared with what would be expected from mixed-age hinds in a commercial herd (Audige et al., 1999; Beatson et al., 2000b). Twenty pregnant hinds were retained until calving with no evidence of foetal loss or abortion, but only 15 hinds reared a calf to weaning which is an unacceptable loss rate. Three of the 20 hinds experienced dystocia, a high dystocia rate compared with those in commercial herds in New Zealand which are reported to average around 0.5-0.8% with a range of 0-9% (Audige et al., 2001; Smyth, 1986). Examination of two foetuses from dams with dystocia and a hind that was euthanased after an assisted delivery showed no pathological or bacteriological evidence of *B. ovis* infection and this high level of dystocia may have been associated with disturbance during calving to blood sample and identify calves. Of the three hinds affected, two were three-year-old hinds that had not calved previously and the third was a four-year-old. The young hinds in this trial were nervous and flighty as they were unaccustomed to handling. Dystocia has not been reported as a feature of *B. ovis* infection in ewes.

A further two calves died between birth and weaning. Post-mortem examination of one calf revealed that it was stillborn but no pathological or bacteriological evidence of *B. ovis* infection was found. The dam of this calf was a primiparous three-year-old and it is possible that the calf death may have been the result of difficult birth. The second calf was estimated to die at four days of age but the carcass was not discovered until several days after death, by which time autolysis was marked. This calf had been blood sampled and ear-tagged soon after birth and it is possible that these procedures may have resulted in mis-mothering and subsequent death by starvation. Thus it is likely that these deaths were due to causes other than *B. ovis* infection. Calf losses of about 4% to 15% during the perinatal/ lactation period have been reported on commercial New Zealand deer farms (Audige et al., 1999; Campbell et al., 2000).

7.5 Conclusions

1) Vaginal *B. ovis* infection of hinds at the time of mating is unlikely to result in significant levels of abortion or perinatal death, which is similar to the disease in ewes.

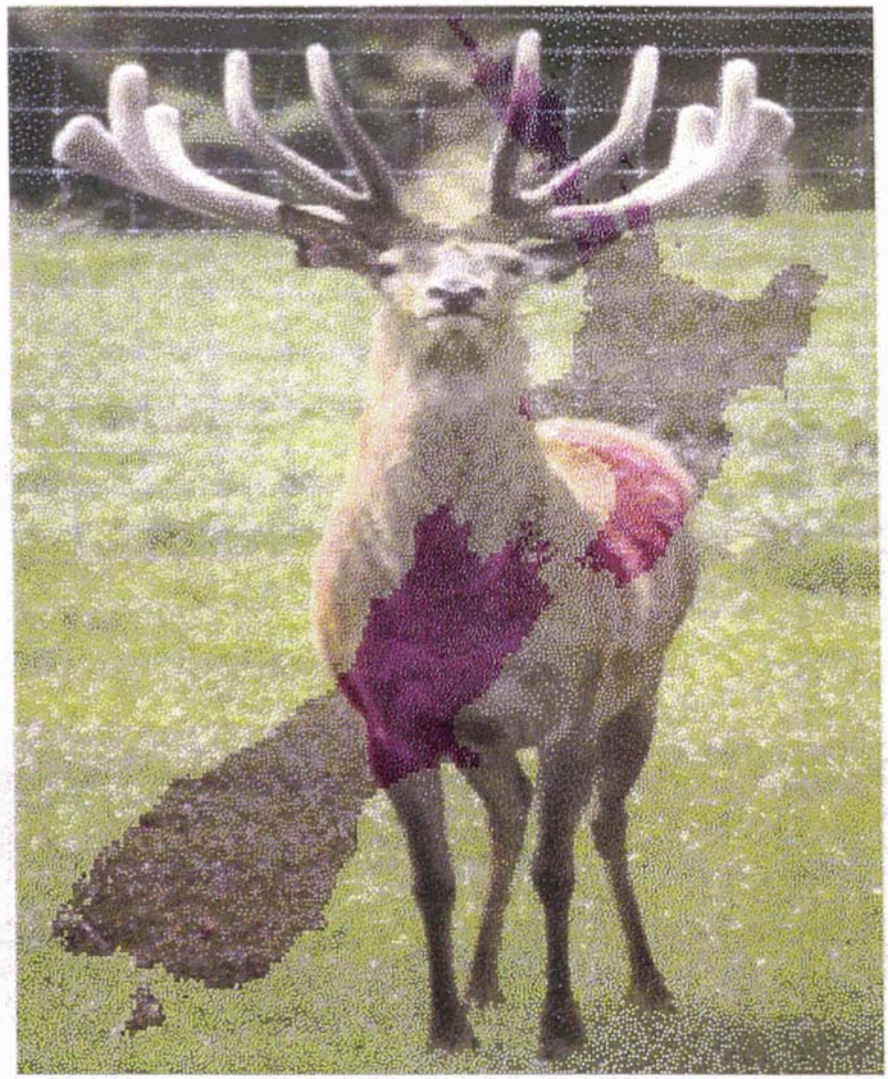
2) Following vaginal inoculation, hinds develop *B. ovis* serum antibodies that are detectable by a commercial CFT and ELISA used for testing sheep sera but titres decrease to negative levels within 120 days of inoculation.

3) Stags can become infected with *B. ovis* by mating vaginally infected hinds, just as rams can become infected by mating vaginally infected ewes.

4) *Brucella ovis* can persist in the vagina of some hinds for at least 337 days after vaginal inoculation.

Chapter Eight

General discussion



A summary of aspects of the material presented in this Ph.D. thesis, entitled "*Brucella ovis* infection in deer" by AL Ridler, has been published in Surveillance, 28 (3), 6-8, 2001.

8.1 Introduction

At the commencement of this Ph.D. study there was limited information about *B. ovis* in the New Zealand farmed deer population. Infection had been identified in stags from five unrelated commercial deer farms (Scott, 1998; Scott, 1999) but it was unknown whether infection was present in stags on other properties. Eighty-eight percent of one group of stags had serological evidence of infection (Anon., 1997a) suggesting that the disease spread readily between stags. It was unknown whether infection of stags would result in stag or hind infertility, abortion storms, or high rates of perinatal calf mortality. The sensitivity of the available *B. ovis* serological tests had not been determined in deer. The high prevalence of infection in some naturally infected herds suggested that this disease could have significant consequences to the deer industry, but the lack of information on the disease in deer meant that further research was required. Therefore this series of studies was undertaken with the broad objectives of establishing the epidemiology, pathogenesis, diagnosis and production effects of the disease in deer, with a view to developing guidelines for the management of this disease both nationally and on the individual farm.

8.2 National prevalence of *B. ovis* infection in deer

During the course of this study, serological data obtained from commercial testing laboratories suggests that the current prevalence of *B. ovis* infection in the New Zealand commercial deer population is low (Section 1.10). Infection has been confirmed on only five properties and since 1998 only one new case has been identified. This was despite a deer slaughter premises targeted surveillance programme involving palpation of the scrotal contents of all stags at slaughter, with any lesions of epididymitis submitted to a diagnostic laboratory for bacteriological culture. However, because transmission of infection can occur from infected rams to in-contact stags (Section 4.2) it is probable that the disease will occur sporadically within the farmed deer population.

8.3 Strain types of *B. ovis* identified by pulsed field gel electrophoresis

Pulsed field gel electrophoresis of 10 ovine field isolates, two cervine field isolates and the Type strain of *B. ovis* demonstrated that two strain types of *B. ovis* are present in New Zealand (Chapter 3). This is the first time that different strains of this organism have been confirmed. Both strains were isolated from both rams and stags, demonstrating that either species can become infected by either strain. The implications, if any, of how the presence of two different strains may impact on the characteristics of the disease are unknown.

8.4 Transmission

Experimentally, it has been demonstrated that transmission of *B. ovis* can occur from infected rams to stags that are in direct contact (Section 4.2). Furthermore, pulsed-field gel electrophoresis of *B. ovis* isolates from sheep and deer (Chapter 3) demonstrated that infection in at least two of the five naturally occurring cases of *B. ovis* in deer was likely to have been from contact with infected rams. This suggests that allowing stags to come into contact with infected rams is a high risk for the transmission of *B. ovis,* highlighting the importance of keeping infected rams separate from stags. It is likely that in the future *B. ovis* infection will continue to occur sporadically within the New Zealand deer population unless deer farmers follow the simple management procedure of keeping rams and stags separate.

West et al., (1999) demonstrated that transmission occurs between stags when in direct contact with one another, so the introduction of an infected stag to a group of stags could result in transmission of infection within the group.

Experimentally, four of eight stags became infected while in contact with infected stags (West et al., 1999) and five of six stags became infected while in contact with infected rams (Section 4.2). In naturally occurring cases of *B. ovis* infection in stags, 30 of 36 stags from one property had serological evidence of infection (Anon., 1997a) and eight of 50, five of 20, and 30 of 60 stags from other properties had *B. ovis* isolated from the epididymes at slaughter (Scott, 1998; Scott, 1999). This demonstrates that the

prevalence of *B. ovis* infection in groups of stags can become high and suggests that there is a relative ease of transmission from rams to stags and between stags.

The high prevalence of infection in young stags in naturally occurring cases of *B. ovis* (Scott, 1998; Scott, 1999) and from transmission experiments between stags (West et al., 1999) and from rams to stags (Section 4.2) would also suggest that stags are highly susceptible to infection. This is further supported by the relative ease of artificial infection experienced during the present studies where five of six stags (Section 4.3) and 12 of 14 stags (Chapter 5) artificially infected by intravenous inoculation shed the organism in semen. These rates of infection are comparable to those following intravenous inoculation of rams where six of eight (Chapter 5), and 80 to 95% of rams (Section 1.2, Table 1.2.1) became infected and shed *B. ovis* in semen. Artificial infection of the mucous membranes of stags resulted in lower rates of infection than intravenous inoculation (Section 4.4). One of four stags inoculated by the conjunctival route, one of four stags inoculated by the nasal route, three of four stags inoculated by the rectal route and none of four stags inoculated by the oral route became infected and shed *B. ovis* in semen. Again, these rates of infection are comparable with infection rates of rams that were experimentally infected by inoculation of mucous membranes (Section 1.2, Table 1.2.1).

It is probable that sexual activity is important in the transmission of *B. ovis* infection. Because sexual activity in stags is seasonal (Lincoln and Guinness, 1973; Lincoln, 1985) it is likely that the majority of transmission occurs during the breeding period. This theory is supported by transmission experiments where infection between stags and from rams to stags occurred during the breeding season (West et al., 1999; Section 4.2). The timing of transmission in the naturally occurring cases of *B. ovis* infection in stags is unknown but it is of interest that infection was detected between May and August in all cases, which is shortly after the breeding season for deer in New Zealand. It has been demonstrated that stags can become infected by inoculation of the conjunctival, nasal and rectal mucous membranes (Section 4.4) and that during the breeding season stags in all-male groups display behaviour that would be considered high risk for transmission of *B. ovis* via each of these routes (Section 4.5). This research has led to the hypotheses that transmission occurs by licking or sniffing the prepuce of an infected ram or stag,

sniffing or licking the anal region of an animal after mounting behaviour with ejaculation onto the perineum, aerosol spray of infected semen or urine, or rectal copulation between stags (Section 4.6). Further work is required to quantify the importance of these various routes of transmission.

It is probable that transmission of *B. ovis* between stags and from rams to stags requires direct contact between animals. Moving six non-infected stags onto paddocks that had only just been vacated by five infected stags on a total of 32 occasions during a five-and-a-half month period, which included the breeding season, did not result in transmission of infection, nor did grazing six non-infected stags in a paddock adjacent to five infected stags for the same time period (Section 4.3). Similarly in sheep, Keogh et al. (1958) alternated two groups of rams between two paddocks each week for a 12-month period without transmission of infection. Furthermore, practical experience suggests that it is possible to keep infected and non-infected rams on a property without transmission of infection (Bruere and West, 1993). This evidence suggests that infected and non-infected animals can be managed on a farm without transmission occurring, provided the groups remain separate.

Experimentally, three of five stags mated separately to vaginally inoculated hinds developed *B. ovis* (Chapter 7), demonstrating that transmission of infection may occur by the venereal route. This suggests that if a breeding stag or stags were infected, joining more than one stag to a group of hinds could result in rapid transmission of infection. It is a common practice for deer farmers to mate a single stag to each group of hinds and they often swap each stag to a separate group of hinds halfway through the mating period (Wilson et al., 1998). It is possible that in this situation, persistence of *B. ovis* in the vagina of hinds could result in transmission between stags and this constitutes a particular risk for farmers who lease stags from other farms for breeding. The persistence of *B. ovis* in the vagina of one of 19 hinds 337 days after vaginal inoculation (Chapter 7) raises the possibility that venereal transmission may occur in the following breeding season, but further experimental work would be required to establish whether this is epidemiologically significant.

8.5 Effects of *B. ovis* infection in deer

8.5.1 Fertility

The most economically significant manifestation of *B. ovis* infection in deer is its effects on the semen quality of stags (Chapter 6). Compared with semen from six non-infected 16-month old stags, semen from 12 artificially infected stags of the same age had reduced sperm motility, an increased number of detached sperm heads and large numbers of leucocytes. Purulent material was grossly visible in the semen of eight of the 12 stags and these were producing semen that would be considered of only fair or poor quality (Carroll et al., 1963; Howard, 1981). The effects of infection on the semen characteristics of stags are similar to those seen in rams infected with *B. ovis* (Jebson et al., 1954; Cameron et al., 1971; Cameron and Lauerman, 1976; Kott et al., 1988).

In commercial sheep flocks where it is standard practice to mate multiple rams to a group of ewes, reduced fertility of individual rams may be masked (Middelberg, 1973) but in deer herds where it is common to mate a single stag to a group of hinds (Wilson, 1998) the effects on reproductive performance may be more severe. Of concern to stud deer farmers is the possibility of infection in sale stags, leading to either wastage of these animals if infection is discovered prior to sale, or dissemination of the disease to other farms if it is not. In rams, this issue is considered by some to be the most economically significant feature of the disease (Bruere, 1973).

For farmers, the consequences of *B. ovis* infection on individual properties are likely to vary depending upon the class(es) of deer that are infected. Infection of breeding stags and stud stags destined for sale to other properties are of concern. However, infection of stags being farmed for velvet antler or meat production is of little economic significance, provided these stags are not used for breeding and as long as transmission of infection to breeding stags does not occur.

8.5.2 Persistence of infection

During these studies it has been demonstrated that 10 of 12 stags infected by intravenous inoculation and three of three stags that became infected by mating vaginally infected hinds stopped shedding *B. ovis* in semen within 300 days of infection (Chapter 5). The organism was not isolated from the reproductive tract of those stags at slaughter 560 to 650 days after infection. The cessation of *B. ovis* shedding in semen, and the failure to isolate the organism from the reproductive tract at slaughter suggests that those stags resolved the infection over time or effectively achieved a "self-cure". Furthermore, 13 stags that apparently resolved the infection were in continuous contact with two infected stags (which did not resolve the infection) for at least 350 days including a breeding season but did not become re-infected and shed *B. ovis* in semen. This suggests that following resolution of infection, stags may be immune to re-infection. It is possible that antimicrobial therapy may hasten the resolution process but this aspect of the infection in stags has not been investigated.

In contrast, six of six rams artificially infected with *B. ovis* by intravenous inoculation continued to shed the organism in semen throughout a 630-day period and *B. ovis* was isolated from the reproductive tracts of these rams at slaughter 649 days after inoculation (Chapter 5). Other researchers have reported that most rams continue to excrete *B. ovis* in semen for periods in excess of two years (Hartley et al., 1955; Buddle, 1956) and this represents an important difference in the characteristics of the disease in rams compared with the disease in stags.

The semen characteristics of stags following resolution of infection showed a dramatic improvement compared with when they were shedding *B. ovis* in semen (Chapter 6). Semen from seven stags that had poor semen quality during the shedding phase showed a significant increase in sperm motility and percentage of morphologically normal sperm following resolution. Semen from all 10 stags in this experiment had very good semen quality (Carroll et al., 1963; Howard, 1981) following resolution of infection compared with only three of the 10 stags during the *B. ovis* shedding phase. This suggests that following resolution of infection the fertility of stags improves to normal levels. Thus if valuable breeding stags become infected, keeping them in a separate group from non-infected stags until the following breeding season should prevent further transmission of

infection and may mean that they will have resolved the infection and be fertile for mating by that time. This demonstrates that a test and slaughter programme in the face of infection is not necessarily the only, or indeed the best, option for some farmers.

However, not all experimentally infected stags resolved *B. ovis* infection within a year. Two of 15 stags continued to shed *B. ovis* in semen for at least 21 months (Chapter 5) and it is unknown whether these stags would have eventually resolved the infection or whether they would continue to shed *B. ovis* in semen indefinitely. Therefore, if the decision were made to keep infected breeding stags until the following breeding season it would be important to determine that they had resolved the infection before using them for mating.

8.5.3 Effects in hinds

Vaginal inoculation with *B. ovis* of 30 hinds on the day of mating and 19 days later did not result in abortion or perinatal mortality (Chapter 7). There was no evidence of placental or foetal infection in six pregnant hinds slaughtered prior to calving. In the first naturally-occurring case of *B. ovis* infection in a stag in New Zealand (O'Neil, 1996), abortion or calf deaths were not reported from the 42 hinds that had been mated to this stag. It is therefore probable that vaginal infection of hinds at the time of mating is of little consequence to the hind. This is similar to the disease in ewes where abortion and perinatal lamb death due to *B. ovis* infection appear to be uncommon. It is possible that inoculation of hinds during mid-pregnancy could result in abortion or perinatal calf mortality, as has been demonstrated with artificial infection of ewes (Osburn and Kennedy, 1966; Ris, 1970, Hughes, 1972a) but in non-experimental circumstances hinds are unlikely to become exposed to the organism during pregnancy.

8.6 Diagnosis of *B. ovis* infection in deer

8.6.1 Scrotal palpation

During the course of these studies only three of 30 stags shedding *B. ovis* in semen had lesions of epididymitis that could be detected by scrotal palpation. Of 17 stags artificially infected by intravenous inoculation (Section 4.3; Chapter 5), two had

palpable lesions of epididymitis. No lesions were detected by scrotal palpation of five stags that had been artificially infected by inoculation of either the conjunctival, nasal or rectal mucous membranes (Section 4.4), although gross examination following slaughter showed a subtle increase in the size of one epididymal tail from each of two stags. Of five stags infected by contact with infected rams (Section 4.2), one had epididymitis detectable by scrotal palpation while none of three stags that became infected by mating vaginally infected hinds did (Chapter 7; Chapter 5). Therefore, on an individual basis, scrotal palpation of live stags is not a sensitive test. Scrotal palpation may detect infection within a herd that has a high prevalence of infection but it would not be an acceptable test for determining the prevalence of the disease in deer, or for declaring a herd free of infection. Scrotal palpation of stags is a dangerous procedure unless it is carried out while they are heavily sedated, or following slaughter.

In contrast, six of six experimentally infected rams developed lesions of epididymitis that could be detected by scrotal palpation (Chapter 5) and more than 30% of naturally infected rams are reported to develop lesions of epididymitis that can be detected by scrotal palpation (Hughes and Claxton, 1968). In sheep, scrotal palpation is an important method of diagnosis on a flock basis (West, 2000).

8.6.2 Serology

Sensitivity

Using the *B. ovis* CFT and ELISA tests commercially available in New Zealand for testing sheep sera and using the cut-off values described for sheep (Section 2.5), within the first 20 to 60 days of infection the CFT and ELISA were 100% sensitive at detecting infection in 17 stags artificially infected by intravenous inoculation (Section 4.3; Chapter 5), five stags that became infected by exposure to infected rams (Section 4.2) and three stags that became infected by mating vaginally inoculated hinds (Chapter 7; Chapter 5). The CFT and ELISA were also 93% and 90% sensitive, respectively, at detecting exposure to infection in 30 hinds 19 days after intravaginal inoculation (Chapter 7) and 94% and 88% accurate respectively at detecting exposure to the organism in 16 stags 11 to 50 days after inoculation of the conjunctival, nasal, oral and rectal mucous membranes (Section 4.5). Therefore it would appear that during the

early stages of infection both of these tests are highly sensitive at detecting infection and at detecting exposure to infection.

However, over time the ability of both the CFT and ELISA to detect infection in stags appears to decrease (Chapter 5). Up to 100 days after infection, the sensitivity of the CFT at detecting infection in 12 stags artificially infected by intravenous inoculation and three stags that became infected by mating vaginally-infected hinds was 100% and the sensitivity of the ELISA ranged from 85 to 100%. From 100 to 300 days after infection, the sensitivity of the CFT decreased to around 30 to 70% and the sensitivity of the ELISA decreased to 10 to 60%. At the same time stags were concurrently resolving the infection.

In another experiment (Section 4.4) the serological titres of two of five stags artificially infected with *B. ovis* by inoculation of either the conjunctival, nasal or rectal mucous membranes had decreased to negative levels in both the CFT and ELISA by 65 days after inoculation despite the presence of *B. ovis* in the reproductive tract at that time. In general, the ELISA had poorer sensitivity than the CFT but it detected additional infected stags that were not detected by the CFT (Chapter 5). Thus the interpretation of the CFT and ELISA tests in parallel is likely to increase the overall sensitivity of serology.

In contrast, the CFT and ELISA were 100% sensitive at detecting infection in six rams throughout a 649-day period (Chapter 5). These rams had been artificially infected with the same dose of *B. ovis* at the same time as 14 stags but unlike serum antibody titres from the stags that declined over time to negative levels, the titres from the rams remained, with one exception, in the positive range.

Despite decreasing sensitivity over time, serological testing is likely to be the most practical and low-cost method of diagnosing *B. ovis* infection in stags. However interpretation would need to be done with caution, with consideration of the objective of testing, the number of stags being tested, the timing of the possible introduction of the infection and any clinical findings. Because case studies and experimental evidence have demonstrated that *B. ovis* infection may occur at a high prevalence within a group

of stags, this would result in a high probability of identifying that infection is present within the group even though some individual infected animals may not be identified. Conversely, infection is more likely to be undetected by serology when testing a single stag that has been infected for some time. Serological testing alone should not be used as the sole criteria to determine resolution of infection.

Guidelines for the New Zealand voluntary *B. ovis* accreditation scheme for sheep recommend that for re-accreditation, scrotal palpation of all rams in a group and serological testing of all rams with lesions detectable by scrotal palpation and a random sample of 20 genitally sound rams is sufficient to confirm a flock is still free of infection (Appendix 2). This guideline was suggested because if infection is present within a flock then it is likely to be detected by scrotal palpation in the first instance, and then serological testing of 20 rams gives a 90% chance of detecting infection if there is a 10% prevalence of infection within the flock (West, 2000). The major benefit of this strategy is that it minimises cost. However, due to the poor sensitivity of scrotal palpation and the declining sensitivity of serology over time described above, this strategy is unlikely to be sufficient to accurately declare a group of stags free from infection. For stags, it would be necessary to blood test all animals in the group or herd.

Specificity

During the course of these studies, 221 serum samples from 104 non-infected deer have been tested in the CFT and 109 samples from 59 non-infected deer have been tested in the ELISA. There has been one false-positive reaction in the CFT (Section 4.3) and none in the ELISA, suggesting a specificity of 99% and 100% respectively. Using the CFT, Kittelberger and Reichel (1998) tested serum from 1498 deer that were presumed to be non-infected, with a specificity of 99.6%. Therefore the specificity of the *B. ovis* CFT and ELISA in deer appears to be high.

During this study, despite attempts to locate a naturally infected herd, the opportunity to investigate and eradicate *B. ovis* infection in a commercial deer herd or an experimentally infected herd did not arise. Therefore further validation of the

effectiveness of serological testing for identifying infected stags in a herd and as a tool for eradication of the disease on a property is required.

8.6.3 Bacteriology

During this study, culture of *B. ovis* from semen has been a reliable method of detecting infection in a total of 30 stags, of which 17 were artificially infected by intravenous inoculation (Section 4.3; Chapter 5), five were artificially infected by inoculation of mucous membranes (Section 4.4), five were infected by contact with infected rams (Section 4.2) and three of which became infected by mating vaginally inoculated hinds (Chapter 7). On no occasion has the organism been cultured from the reproductive tract of a stag when it was not previously isolated from the semen from that stag. Therefore, culture of *B. ovis* from the semen of stags appears to be a sensitive method of diagnosing the infection. However, because the organism does not grow well in the presence of contamination and because on rare occasions it may not be possible to isolate the organism from the semen of some animals at some times, in equivocal cases collection and culture of two semen samples may be necessary. Also, because semen collection and culture requires some expertise it is a more time-consuming and expensive method of diagnosing *B. ovis* infection than serology.

At slaughter, *B. ovis* has been isolated from the reproductive tracts of a total of 10 stags, five of which were infected by contact with infected rams (Section 4.2), four of which were infected by inoculation of mucous membranes (Section 4.4) and one which was artificially infected by intravenous inoculation (Chapter 5). The organism was isolated from the epididymes of five of these 10 stags, the seminal vesicles of nine and the ampullae of five. Therefore it appears that no single organ within the reproductive tract of stags consistently becomes infected with *B. ovis* and if culture of the reproductive organs is selected as a diagnostic method all three organs should be sampled. In rams, *B. ovis* was isolated from the epididymes of six of six artificially infected rams, the seminal vesicles of five and the ampullae of four (Chapter 5). Other researchers have isolated the organism from between 50 to 100% of each of these organs (Section 1.3, Table 1.3.2).

8.6.4 Pathology

Gross pathology

As previously described, lesions of epididymitis that could be detected by scrotal palpation were present in three of 30 infected stags (Section 4.2; Chapter 5). At slaughter a further two stags that had become infected by exposure to infected rams had foci of white caseous material within the epididymes (Section 4.2), two other stags that had been artificially infected by inoculation of mucous membranes had a subtle increase in the size of the tail of one epididymis that could be detected on examination after slaughter (Section 4.4) and one stag that had become infected by mating vaginally infected hinds had an adhesion between the testes and the epididymal tail (Chapter 5). In contrast, six of six rams artificially infected by intravenous inoculation had lesions of epididymitis that could be detected by scrotal palpation and that were visible on examination after slaughter (Chapter 5). These lesions, consisting of epididymal enlargement, increased intraepididymal connective tissue, adhesions between the tunica vaginalis and/or foci of caseous material, were similar to those described by previous researchers (Jebson et al., 1955; Kennedy et al., 1956; Blasco, 1990).

Grossly, lesions were not visible in the seminal vesicles or ampullae of any of the 30 infected stags or any of six infected rams, which is consistent with the observations of Kennedy et al. (1956) and Foster et al. (1987) who did not find gross lesions in these organs from rams infected with *B. ovis*.

Histopathology

Histopathological examination of the epididymes, seminal vesicles and ampullae of five stags that became infected from exposure to infected rams and were slaughtered within 30 days of infection (Section 4.2) showed prominent lesions consisting of large aggregates of lymphocytes within the lamina propria of all three organs and spermatic granulomas and intraepithelial cysts within the epididymes. Similar lesions were present within the reproductive tracts from five stags artificially infected by inoculation of mucous membranes and slaughtered within 65 days of infection (Section 4.4), although these lesions did not appear to be as prominent. The histological lesions present in the reproductive tracts of 12 stags infected by intravenous inoculation and three stags that became infected by mating vaginally-infected hinds that were

slaughtered 560 to 650 days after infection were mild and consisted of occasional small foci of five to 10 lymphocytes, although all but one of these stags had resolved the infection prior to slaughter (Chapter 5). This would suggest that in stags the cellular changes in the reproductive tract in response to *B. ovis* infection may regress over time, which may also be a function of resolution of infection.

In contrast, the histological lesions present in the reproductive tract of rams infected by intravenous inoculation and slaughtered 649 days later were prominent and relatively severe (Chapter 5). The lesions were similar to those described by Kennedy et al. (1956), Biberstein et al. (1964) and Foster et al. (1987). They were also similar to those seen in the reproductive tracts of stags that had been infected for less than a month (Section 4.2) as described above.

Histological lesions suggestive of or consistent with *B. ovis* infection were present in the epididymes, seminal vesicles and ampullae of six stags infected by inoculation of mucous membranes (Section 4.4) and two stags infected by intravenous inoculation (Chapter 5) that had not had *B. ovis* isolated from the semen or reproductive tract at any time. This would suggest that *B. ovis* had localised in the reproductive tract but the infection had resolved before semen collection. Histopathological examination of the reproductive tracts of stags following slaughter may be a useful tool to support a diagnosis of previous exposure to the organism.

8.7 Management of *B. ovis* infection in deer

Results of research in this thesis suggests that the methods of dealing with *B. ovis* infection in stags needs to differ from those of rams, where the standard practice is to cull infected rams as soon as the diagnosis of *B. ovis* infection is made (West and Bruce, 1991; Reichel et al., 1994). It has already been discussed that infection of stags farmed for velvet antler or meat production is of little economic significance, provided transmission to breeding stags does not occur, and therefore slaughtering non-breeding stags as soon as a diagnosis of *B. ovis* is made may not be necessary. If strict separation between deer groups can be maintained, keeping infected stags separate from others should contain the disease, making culling unnecessary.

Infection of breeding stags is of greater concern because of the effects on semen quality (Chapter 6) and therefore the possible impact on herd reproductive performance. It is probable that rapid transmission of infection could occur during mating due to venereal transmission (Chapter 7). It is therefore important that infected stags are identified prior to mating and not used for mating during that season. It has been demonstrated that the majority of stags stop shedding *B. ovis* in semen within a year of becoming infected and that the organism cannot be isolated from the reproductive tract at slaughter (Chapter 5), suggesting resolution or "self-cure" of the infection in most stags. Furthermore, following the apparent resolution of infection, stags do not appear to become re-infected and the semen quality is at a level considered acceptable for breeding (Chapter 6). Thus the immediate culling of infected breeding stags, particularly valuable stags, appears not to be warranted. An alternative course of action may be to keep the infected stag or stags separate until the following breeding season and then undertake semen evaluation and bacteriological culture. If stags are no longer shedding *B. ovis* in semen and have acceptable semen quality then there is no reason why they should not be used for mating.

This is the first study investigating the transmission, effects and diagnosis of *B. ovis* infection in deer. It has highlighted that many aspects of the disease in deer are similar to those in sheep but there are also some important differences, especially with regard to the long-term persistence of the infection. These findings could form the basis for the control of this disease in the deer industry.

Appendix 1

Raw serological data for experimental deer and rams

Table A1.1 *B. ovis* CFT and ELISA titres from sera from six rams artificially infected with *B. ovis* by intravenous inoculation on Day 0 (refer Section 4.2).

	DAYS AFTER INOCULATION											
	0		14		26		43		141		173	
RAM ID	CFT	ELISA	CFT	ELISA	CFT	ELISA	CFT	ELISA	CFT	ELISA	CFT	ELISA
I	0	0	1/64	158	3/256	265	1/32	192	1/64	482	1/128	478
II	0	0	3/128	190	2/256	409	1/256	361	2/64	356	2/64	402
III	0	0	2/256	375	1/256	454	1/64	390	2/128	641	3/32	631
IV	0	0	2/512	293	2/512	398	3/32	409	3/128	725	3/32	695
V	0	0	3/256	355	1/2048	532	3/64	504	2/128	754	3/64	667
VI	0	0	3/512	247	3/128	187	2/64	217	2/32	337	1/32	420

Table A1.2 *B. ovis* CFT titres from sera from six stags that were in contact with six *B. ovis*-infected rams from Day 0 to Day 131 (refer Section 4.2)

	DAYS AFTER EXPOSURE TO RAMS					
STAG ID	0	20	55	92	124	131
1	0	0	0	0	3/128	2/512
2	0	0	0	0	4/64	1/512
3	0	0	0	0	2/64	1/64
4	0	0	0	0	3/64	1/128
5	0	0	0	0	3/16	4/32
6	0	0	0	0	0	0

Table A1.3 *B. ovis* CFT titres from sera from six stags that were artificially infected with *B. ovis* by intravenous inoculation on Day 0 (refer Section 4.3).

	DAYS AFTER INOCULATION									
Stag ID	**0**	**14**	**26**	**43**	**105**	**118**	**174**	**189**	**203**	**217**
1	0	3/16	1/64	4/128	1/32	1/32	1/16	3/8	3/8	3/16
2	0	1/128	3/512	4/256	4/32	3/32	2/16	1/16	3/8	1/16
3	0	4/64	3/256	2/32	4/64	2/64	2/16	1/16	1/8	2/16
4	0	3/128	3/128	3/64	1/64	1/32	2/16	1/16	1/8	2/16
5	0	3/128	3/256	3/128	4/32	3/128	2/32	1/32	2/16	1/16
6	0	3/256	3/256	1/64	0	0	0	0	0	0

Table A1.4 *B. ovis* ELISA titres from sera from six stags that were artificially infected with *B. ovis* by intravenous inoculation on Day 0 (refer Section 4.3).

	DAYS AFTER INOCULATION									
Stag ID	**0**	**14**	**26**	**43**	**105**	**118**	**174**	**189**	**203**	**217**
1	0	246	179	288	334	301	248	183	258	62
2	0	674	589	402	397	430	184	198	126	160
3	0	417	435	228	493	521	289	308	346	351
4	0	415	251	311	340	280	255	173	150	59
5	0	364	334	367	568	562	506	394	231	335
6	0	297	313	200	40	27	3	5	2	2

Table A1.5 *B. ovis* CFT and ELISA titres from sera from 16 stags that were artificially infected with *B. ovis* on Day 0 by inoculation of either the conjunctival (C), nasal (N), oral (O) or rectal (R) mucous membranes (refer Section 4.5).

	DAYS AFTER INOCULATION											
	0		11		24		36		56		65	
STAG ID	CFT	ELISA	CFT	ELISA	CFT	ELISA	CFT	ELISA	CFT	ELISA	CFT	ELISA
C1	0	3	2/32	33	2/128	95	4/128	514	1/16	135	2/4	85
C2	0	7	1/64	87	1/128	173	2/64	423	4/16	184	1/16	107
C3	0	5	1/16	77	1/32	695	1/64	290	0	109	1/4	58
C4	0	3	1/32	85	2/32	111	4/32	217	1/4	131	2/4	64
N1	0	4	3/64	44	2/64	61	3/64	182	0	55	0	12
N2	0	6	1/32	28	1/64	39	1/64	110	1/8	47	1/8	42
N3	0	3	1/128	108	3/32	93	1/128	232	1/8	150	2/4	65
N4	0	3	2/64	75	1/64	111	3/64	213	3/4	108	1/4	52
O1	0	2	1/4	19	1/32	49	2/32	136	0	32	1/4	29
O2	0	5	1/4	10	3/8	37	1/8	28	0	12	0	10
O3	0	6	0	7	2/4	36	3/4	27	0	20	0	18
O4	0	8	0	3	1/16	33	2/32	123	0	34	0	18
R1	0	2	1/8	25	4/128	180	4/128	507	1/32	268	1/32	208
R2	0	4	1/8	19	2/128	102	3/128	456	4/16	269	1/32	171
R3	0	2	3/16	142	4/128	177	3/128	518	3/64	480	3/16	210
R4	0	3	1/8	85	2/128	153	4/128	445	4/16	261	3/16	132

Table A1.6 *B. ovis* CFT titres from sera from 14 stags that were artificially infected with *B. ovis* by intravenous inoculation on Day 0 (refer Chapter 5).

	DAYS AFTER INOCULATION																			
STAG ID	**0**	**25**	**55**	**83**	**103**	**138**	**166**	**200**	**223**	**235**	**263**	**291**	**342**	**437**	**459**	**467**	**493**	**524**	**559**	**592**	**630**
803	0	1/128	1/16	4/128	2/32	3/32	4/32	2/16	2/16	2/8	1/8	4/8	0	1/4	1/8	1/4	3/8	2/16	3/128	2/32	1/16
826	0	4/64	2/16	3/16	0	0	0	0	0	0	0	0	0	0	0	1/4	0	0	ns	0	0
827	0	1/128	2/16	1/128	4/32	4/32	4/32	1/32	4/8	4/4	3/8	1/8	0	1/8	4/16	3/16	4/16	3/16	ns	2/8	2/8
842	0	1/128	1/32	3/32	2/16	1/16	4/16	2/4	0	0	1/4	4/4	0	0	1/4	1/4	0	1/8	4/16	0	0
852	0	2/32	1/64	1/128	2/32	1/8	1/32	2/4	0	0	1/4	2/4	0	0	0	0	0	0	ns	0	0
854	0	2/32	1/32	1/64	4/128	1/128	4/128	1/32	2/16	4/8	1/16	0	0	1/8	1/16	4/8	1/8	1/8	1/16	2/8	0
855	0	1/32	1/32	1/128	2/64	1/8	1/16	2/4	0	0	2/4	1/4	0	0	0	0	0	ac	ns	0	0
860	0	2/64	4/16	1/32	2/8	1/16	1/32	2/8	2/8	0	1/4	1/4	0	0	0	0	0	1/4	ns	0	0
861	0	1/128	1/16	1/16	3/4	NS	0	0	0	0	0	0	0	0	0	1/4	0	1/4	ns	0	0
864	0	1/128	1/32	3/32	4/16	1/8	4/16	3/16	2/16	4/4	1/8	2/8	0	0	1/4	0	0	0	ns	0	0
865	0	1/128	2/16	1/64	1/16	1/8	4/16	0	3/4	0	0	0	0	0	0	0	0	0	2/16	0	0
866	0	2/128	1/128	4/128	1/128	2/64	4/128	3/32	2/32	4/16	3/16	4/8	0	3/4	1/16	4/8	1/8	1/16	ac	0	0
867	0	3/64	1/32	2/64	4/8	2/4	4/4	0	2/4	0	0	0	0	0	0	0	0	0	ns	0	0
869	0	1/64	1/32	4/128	4/128	1/32	3/16	4/8	2/16	1/8	2/8	4/8	0	1/4	1/8	4/4	2/4	1/8	ac	0	0

NS: no sample
ac: anticomplementary reaction
ns: non-specific reaction

Table A1.7 *B. ovis* ELISA titres from sera from 14 stags that were artificially infected with *B. ovis* by intravenous inoculation on Day 0 (refer Chapter 5).

	DAYS AFTER INOCULATION																			
STAG ID	**0**	**25**	**55**	**83**	**103**	**138**	**166**	**200**	**223**	**235**	**263**	**291**	**342**	**437**	**459**	**467**	**493**	**524**	**559**	**592**	**630**
803	2	171	160	333	384	156	197	118	93	66	116	58	48	45	57	77	75	177	162	117	212
826	5	330	179	72	97	72	32	40	31	25	17	12	34	55	18	19	34	38	22	33	39
827	6	221	234	255	489	274	294	149	147	123	74	62	59	92	153	220	173	195	62	87	185
842	3	219	282	184	297	125	127	69	58	42	58	43	46	45	33	67	81	104	41	49	197
852	3	195	286	127	317	135	160	80	66	41	35	32	50	52	52	56	75	81	37	84	156
854	4	131	288	108	641	354	306	188	152	175	150	75	23	57	60	62	69	120	37	40	138
855	2	167	284	246	179	89	105	57	40	24	60	32	24	25	18	15	31	39	9	16	36
860	5	175	245	62	160	129	132	57	35	29	41	22	33	45	26	34	36	44	10	15	40
861	6	202	223	52	118	NS	48	25	21	13	8	14	23	42	21	27	32	43	14	16	43
864	8	262	304	122	280	154	244	128	104	109	47	90	38	24	26	31	27	51	15	19	42
865	3	324	191	139	213	46	105	47	31	18	14	13	21	31	17	23	44	48	12	18	32
866	4	178	461	296	486	271	360	171	164	133	68	88	116	93	97	97	94	110	47	51	101
867	4	268	291	124	337	71	88	49	31	18	25	13	15	20	19	20	21	32	10	12	23
869	2	223	309	358	549	232	247	118	83	107	74	69	52	42	35	71	39	73	29	25	56

NS: no sample

Table A1.8 *B. ovis* CFT titres from sera from eight rams artificially infected with *B. ovis* by intravenous inoculation on Day 0 (refer Chapter 5).

	DAYS AFTER INOCULATION																			
RAM ID	**0**	**25**	**55**	**83**	**103**	**138**	**166**	**200**	**223**	**235**	**263**	**291**	**342**	**437**	**459**	**467**	**493**	**524**	**559**	**592**	**630**
55	0	2/32	3/32	4/4	1/4	1/4	0	2/8	0	4/4	1/4	0	0	0	0	0	0	0	0	0	0
56	0	1/64	1/64	3/64	1/32	3/128	4/128	2/128	4/16	4/16	3/32	1/32	4/32	3/32	3/128	3/128	4/128	4/128	4/128	4/128	4/128
57	0	3/128	3/128	4/128	2/128	1/128	3/128	4/128	4/64	1/128	4/128	2/128	3/32	1/64	1/128	1/128	4/64	4/128	4/128	1/128	4/32
58	0	3/128	3/128	4/128	3/128	3/128	4/128	4/128	3/128	3/128	3/128	4/128	4/128	4/128	4/128	4/128	4/128	4/128	4/128	3/128	1/128
59	0	4/128	1/128	1/128	4/16	2/32	4/128	2/128	2/32	4/16	1/64	1/64	4/128	2/8	1/16	3/16	4/16	1/32	4/16	2/16	4/4
60	0	4/128	2/128	1/128	3/32	2/128	4/128	2/128	2/32	4/16	3/64	4/128	4/32	4/64	4/128	4/128	4/128	4/128	4/128	4/128	3/32
61	0	4/128	1/64	4/16	4/8	2/16	1/64	2/128	4/128	4/128	4/128	3/64	4/128	1/32	4/128	4/128	4/128	4/128	4/128	2/64	2/16
69	0	2/128	1/32	1/128	4/32	3/16	4/64	4/128	4/32	4/32	1/128	4/128	1/64	2/8	4/32	2/32	1/64	4/32	1/64	2/16	4/8

Table A1.9 *B. ovis* CFT titres from sera from eight rams artificially infected with *B. ovis* by intravenous inoculation on Day 0 (refer Chapter 5).

	DAYS AFTER INOCULATION																			
RAM ID	**0**	**25**	**55**	**83**	**103**	**138**	**166**	**200**	**223**	**235**	**263**	**291**	**342**	**437**	**459**	**467**	**493**	**524**	**559**	**592**	**630**
55	8	137	234	64	48	118	114	57	72	41	38	61	58	70	33	43	52	46	17	28	39
56	5	158	306	252	222	321	378	230	487	400	250	340	405	385	556	456	320	529	417	569	2262
57	3	227	480	417	393	396	369	266	536	442	670	555	433	368	496	444	396	479	382	313	1275
58	3	241	411	412	411	427	396	305	716	558	331	465	827	438	635	495	473	636	514	528	1988
59	4	262	385	313	281	332	363	239	643	485	342	652	580	338	387	388	308	352	184	185	920
60	6	323	380	391	307	438	399	297	733	604	566	88	637	467	711	521	486	630	520	606	2332
61	2	142	257	109	137	208	309	99	915	610	368	524	637	379	531	438	361	451	366	359	1373
69	3	96	289	302	305	340	405	166	562	505	348	583	630	363	517	409	367	394	365	310	1715

Table A1.10 *B. ovis* CFT titres from sera from three stags that became infected on approximately Day 0 by mating hinds that had received a vaginal inoculation of *B. ovis* (refer Chapters 5 and 7).

	DAYS AFTER INFECTION															
STAG ID	**0**	**55**	**77**	**134**	**146**	**174**	**202**	**253**	**350**	**371**	**379**	**405**	**435**	**470**	**504**	**542**
833	0	1/64	4/32	0	0	0	0	0	0	0	0	0	0	ns	0	0
839	0	2/8	1/32	2/8	0	0	4/8	0	0	0	0	0	0	ns	0	0
850	0	4/8	1/64	0	0	0	0	0	0	0	0	0	NS	ns	0	0

NS: no sample
ns: non specific reaction

Table A1.11 *B. ovis* ELISA titres from sera from three stags that became infected on approximately Day 0 by mating hinds that had received a vaginal inoculation of *B. ovis* (refer Chapters 5 and 7).

	DAYS AFTER INFECTION															
STAG ID	**0**	**55**	**77**	**134**	**146**	**174**	**202**	**253**	**350**	**371**	**379**	**405**	**435**	**470**	**504**	**542**
833	3	159	229	3	22	16	9	51	84	60	84	72	89	36	81	140
839	3	72	108	64	35	17	45	23	16	14	14	25	44	9	14	17
850	5	86	160	40	23	20	12	22	48	26	34	44	NS	14	18	94

NS: no sample

Table A1.12 *B. ovis* CFT titres from sera from 30 hinds that were artificially infected with *B. ovis* by vaginal inoculation on Days 0 and 19 (refer Chapter 7).

	DAYS AFTER FIRST INOCULATION										
HIND ID	**0**	**19**	**55**	**85**	**120**	**152**	**183**	**216**	**337**	**378**	**420**
1=723	0	3/4	4/16	0	Cull						
2=731	0	3/32	2/4	0	Cull						
3=533	0	4/128	4/4	1/8	Cull						
4=906	0	1/16	0	0	Cull						
5=252	0	2/32	0	0	Cull						
6=10	0	4/128	4/32	3/8	3/4	4/4	2/8	Cull			
7=12	0	1/128	4/128	2/32	3/8	3/4	1/16	Cull			
8=2	0	0	0	0	0	0	0	Cull			
9=903	0	1/64	2/16	1/8	0	0	0	Cull			
10=916	0	2/128	1/8	1/8	0	0	1/8	Cull			
11=628	0	1/16	3/4	0	0	0	0	0	Cull		
12=919	0	2/32	4/4	1/4	0	0	0	0	2/4	1/4	Cull
13=718	0	4/128	1/8	2/4	0	0	0	0	0	4/16	Cull
14=17	0	2/64	1/8	1/8	0	0	0	0	0		0
15=411	0	3/32	2/16	3/4	0	0	0	0	0		0
16=533	0	4/128	4/4	1/8	0	0	0	0	0		0
17=616	0	2/64	4/4	0	0	0	0	0	0		0
18=618	0	3/64	4/32	1/8	0	0	0	0	0		0
19=711	0	2/32	3/4	0	0	0	0	0	0		0
20=720	0	1/128	2/8	3/8	0	0	0	0	0		0
21=722	0	2/128	2/8	1/8	0	0	0	0	0		0
22=732	0	3/8	0	0	0	0	0	0	0		0
23=902	0	2/16	0	0	0	0	0	0	0		0
24=904	0	3/8	2/4	0	0	0	0	0	0		0
25=905	0	1/16	0	0	0	0	0	0	0		0
26=908	0	1/32	1/4	0	0	0	0	0	0		0
27=910	0	3/8	0	0	0	0	0	0	0		0
28=914	0	2/128	3/4	0	0	0	0	0	0		0
29=920	0	2/32	1/8	1/4	0	0	0	0	0		0
30=922	0	1/16	4/4	1/4	0	0	0	0	0		0

Table A1.13 *B. ovis* ELISA titres from sera from 30 hinds that were artificially infected with *B. ovis* by vaginal inoculation on Days 0 and 19 (refer Chapter 7).

	DAYS AFTER FIRST INOCULATION										
HIND ID	**0**	**19**	**55**	**85**	**120**	**152**	**183**	**216**	**337**	**378**	**420**
1=723		106	70	30	18	Cull					
2=731		149	37	23	11	Cull					
3=533		225	49	39	23	Cull					
4=906		115	32	24	9	Cull					
5=252		155	45	28	14	Cull					
6=10		405	159	99	163	33	51	Cull			
7=12		382	217	170	185	87	54	Cull			
8=2		131	30	25	28	9	6	Cull			
9=903		154	86	58	41	12	18	Cull			
10=916		163	93	55	44	17	41	Cull			
11=628		171	41	21	10	5	3	9	Cull		
12=919		98	45	35	53	23	64	65	57	61	Cull
13=718		256	85	29	32	14	9	15	11	259	Cull
14=17		237	89	66	58	31	27	122	11		5
15=411		181	74	39	52	12	8	18	10		4
16=503		84	28	29	34	5	8	10	11		9
17=616		137	56	26	23	5	7	9	20		5
18=618		179	110	79	48	20	14	44	14		5
19=711		142	49	25	17	4	7	8	11		7
20=720		198	94	50	29	11	15	13	10		3
21=722		161	63	27	10	6	3	13	6		7
22=732		102	20	18	16	5	3	7	5		3
23=902		67	17	30	10	5	5	21	7		4
24=904		107	13	18	11	5	2	20	5		4
25=905		83	21	27	16	7	8	12	7		5
26=908		151	32	21	16	13	26	80	6		4
27=910		37	30	18	5	4	2	16	2		9
28=914		224	103	23	31	10	12	95	9		7
29=920		98	41	65	23	13	10	45	10		5
30=922		57	36	30	12	5	3	14	4		8

Table A.1.14 *B. ovis* CFT and ELISA titres from sera collected from 15 calves at 1-3 days, 10-15 weeks and 22-27 weeks after birth, whose dams received an intravaginal inoculation of B. ovis on Day 0 and 19 of mating (refer Chapter 7).

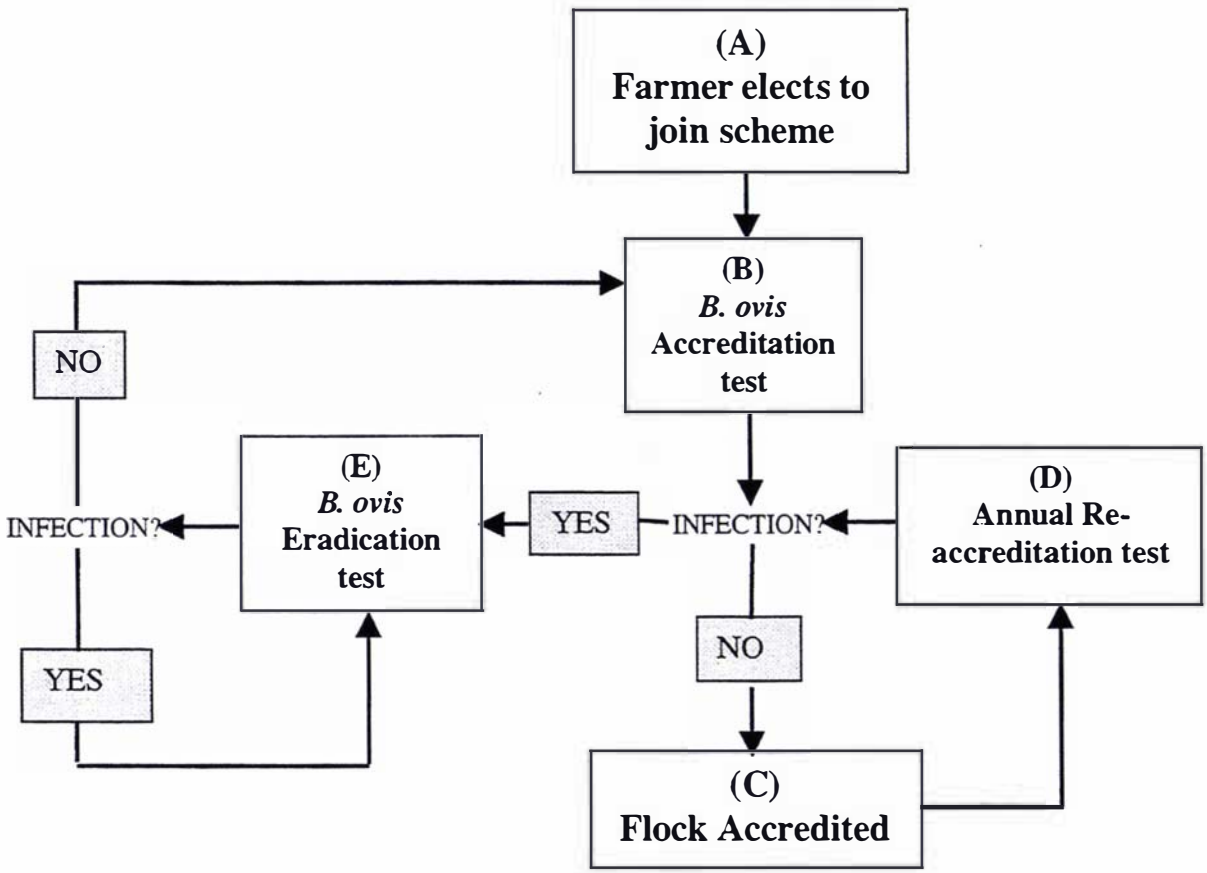
	Time after birth					
Calf ID	**1-3 days**		**10-15 weeks**		**22-27 weeks**	
	CFT	**ELISA**	**CFT**	**ELISA**	**CFT**	**ELISA**
228	0	18	0	2	0	3
229	0	12	0	1	0	2
230	0	121	0	4	0	2
231	0	27	0	2	0	2
232	1/8	112	0	2	0	1
233	0	129	0	2	0	7
234	AC	108	0	2	0	5
235	2/4	200	0	3	0	3
236	0	31	0	1	0	3
237	0	152	0	2	0	1
238	3/32	381	0	5	0	2
239	1/16	232	0	2	0	2
240	1/16	307	0	7	0	4
241	0	56	died	died	-	-
NT1	ns	ns	0	2	0	1
NT2	ns	ns	0	5	0	7

AC: anti-complementary
ns: no sample

Appendix 2

A summary of the New Zealand voluntary *B. ovis* accreditation scheme for sheep

Flow chart summary of scheme



Notes to accompany flow chart

(A) Owner elects to join the scheme

This is an industry based scheme and the participation of ram flock owners is voluntary. The veterinary practitioner is responsible for carrying out all the testing of rams for *B. ovis* and certifying the flock when it is free. All costs of examinations and testing should be borne by the owner.

(B) Accreditation test

To fulfil the requirements for accreditation the veterinarian is to:

a) Check that no new rams, other than from accredited free flocks have been introduced within the previous two months.

b) Palpate the scrotal contents and blood sample and serologically test all rams and teasers over the age of 15 months and rams under 15 months that have been used for breeding.

c) Palpate the scrotal contents of all sale rams within three months of sale for breeding purposes. Blood sample and serologically test any with lesions of epididymitis.

(C) Flock accredited

The veterinarian issues a Certificate of Accreditation (available from approved laboratories) to flock owner. Accreditation will be valid for a period of one year from the date of testing.

(D) Annual reaccreditation test

a) All stud rams and teasers over the age of 15 months and stud rams less than 15 months of age which have been used for mating: scrotal palpation and blood samples for serology.

b) Commercial rams over the age of 15 months: scrotal palpation of all rams, blood samples from the whole flock or 20 rams, whichever is the least, and any ram with epididymitis.

c) Scrotal palpation of all sale rams within three months of sale for breeding purposes. Blood sample any with lesions of epididymitis.

(E) Eradication testing

Should *B. ovis* infection be diagnosed and should the owner wish to proceed towards accreditation, then a *B. ovis* eradication programme should be implemented on the farm under the supervision of the veterinarian. The eradication programme should be adapted to the particular flock circumstances on the property concerned.

The flock may be accredited when:

a) All rams and teasers 15 months of age and over, and any younger than 15 months which have been used for mating, have had two consecutive negative blood tests no less than 60 days and not more than 180 days apart.

b) All sale rams have undergone scrotal palpation within three months of sale for breeding purposes, and any with lesions of epididymitis have been negative to serology.

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